Identification and analysis of *Dictyostelium discoideum* microtubule associated proteins

Dissertation

zur

Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

dem

Fachbereich Biologie

der Philipps-Universität Marburg

vorgelegt von

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aus München

Marburg/Lahn 2006

Vom Fachbereich Biologie der Philipps-Universität Marburg als Dissertation angenommen am: 05. September 2006

Tag der mündlichen Prüfung: 27. November 2006

Erstgutachter: Prof. Dr. Michael Bölker
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Erklärung

Ich versichere, dass ich meine Dissertation selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

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Marburg, im Oktober 2006 Katrin Veronika Koch

Die Untersuchungen zur vorliegenden Arbeit wurden von April 2003 bis August 2006 im Labor von Prof. Dr. Manfred Schliwa am Adolf-Butenandt-Institut für Zellbiologie der Ludwig-Maximilians-Universität in München unter der Betreuung von Prof. Dr. Ralph Gräf durchgeführt. Betreuer an der Philipps-Universität Marburg war Prof. Dr. Michael Bölker.

Part of this work has been published:

Koch, K.V., Reinders, Y., Ho, T.H., Sickmann, A., Gräf, R., 2006. Identification and isolation of *Dictyostelium* microtubule-associated protein interactors by tandem affinity purification. European Journal of Cell Biology. 85 (9-10).

Talks:
Koch, K.V. 2005. *Dictyostelium discoideum* - a model organism suitable for studying Microtubule associated Protein Interactors by Tandem Affinity Purification; At: Department of Botany Seminar. University of Toronto, Toronto, Canada.

Koch, K.V. 2005. Identification of *Dictyostelium* microtubule associated protein interactors by Tandem affinity purification; At: 5th Biomedical Center (BMC) Seminar Series. LMU, München, Germany.

Meeting abstracts:
Koch, K.V., Reinders, Y., Sickmann, A., Gräf, R. 2006. Tandem Affinity Purification in *Dictyostelium*: Search for Interactors of EB1 and DdCP224 at the Centrosome and the Microtubule Plus-ends, pp. 119 Jahrestagung DGZ, Vol. 85S1. European Journal of Cell Biology, TU Braunschweig.

Koch, K.V., Reinders, Y., Sickmann, A., Gräf, R. 2005a. Tandem Affinity Purification in *Dictyostelium*: Search for Interactors of EB1 and DdCP224 at the Centrosome and the Microtubule Plus-ends. The American Society for Cell Biology 45th Annual Meeting, Vol. 16. Molecular Biology of the Cell Supplement, Moscone Center, San Francisco.

Koch, K.V., Reinders, Y., Sickmann, A., Gräf, R. 2005c. Screen for interactors of DdEB1 and DdCP224 at the centrosome and the microtubule tip in *Dictyostelium* SFB 413 Munich Symposium on Cell Dynamics: From Molecular Structure to Cellular Motility, München/Martinsried.

Koch, K.V., Reinders, Y., Sickmann, A., Gräf, R. 2005b. Screen for interactors of DdEB1 and DdCP224 at the centrosome and the microtubule tip in *Dictyostelium* EMBO Workshop Centrosomes and Spindle Pole Bodies. EMBO, EMBL Heidelberg.

Koch, K.V., Wagner, Y., Hestermann, A., Rehberg, M., Sickmann, A., Gräf, R. 2005d. Screening for interactors of *Dictyostelium* EB1 and the XMAP215 family member DdCP224 at the microtubule tip and the centrosome, pp. 51-52 Jahrestagung DGZ, Vol. 84S1. European Journal of Cell Biology, Heidelberg.

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Abbreviations

aa	Amino acid
ATP	Adenosine-5'-triphosphate
BCIP	Bromo-chloro-indolyl phosphate
bp	Base pairs
BSA	Bovine serum albumin
C'-	Carboxy terminal
cDNA	Complementary DNA
Dd	*Dictyostelium discoideum*
D	Daltons
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide triphosphate
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetraacetic acid
EGTA	Ethyleneglycol-bis-(2-aminoethylether)-N,N'-tetraacetic acid
FITC	Flourescein isothiocyanate
g	Gravity
GFP	Green fluorescent protein
GTP	Guanosin-5'-triphosphate
H_2O	Distilled water
IPTG	Isopropyl-β-thiogalactopyranoside
kbp	Kilo base pairs
kDa	Kilo Daltons
M	Molarity [mol/l]
MM	Molecular mass [D]
MAP	Microtubule associated protein
MOPS	Morpholinopropanesufonic acid
mRNA	Messenger ribonucleic acid
MT	Microtubules
MTOC	Microtubule organizing center
MW	Molecular weight

N' -	Amino terminal
NADH	Nicotine adenine dinucleotide
NBT	Nitroblue-tetrazolium chloride
OD_x	Optical density at wavelength x [nm]
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
pH	Negative decadic logarithm of proton concentration
PI	Protease inhibitor
PIPES	Piperazine-N,N'-bis-[2-ethanesulfonic acid]
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SPB	Spindle pole body
TAP	Tandem affinity purification
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N,N' -tetramethylenediamine
Tris	Tris-hydroxymethyl-ammoniumethane
Triton-X-100	T-Octylphenoxypolyethoxethanol
Tween 20	Polyoxyethylene-sorbianemonolaureate
U	Units
UTR	Untranslated region
v/v	Volume per volume
w/v	Weight per volume
wt	Wild-type
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Unless stated otherwise, SI-units, derived units and the decimal multiple of SI-units were used.

Summary

The microtubule cytoskeleton and its dynamic ends are crucial for many cellular functions throughout the whole cell cycle. Microtubule associated proteins (MAPs) are known to interact with other proteins to fulfill these complex functions in balancing the dynamic instability of microtubules as well as anchoring microtubules at the cell cortex, guiding transport along them and controlling mitosis at the centrosome. Deficient function of these proteins leads to severe defects, including cancer. A major part of our understanding of these processes is knowing the proteins associated with the complexes at either end of microtubules.

To identify new members of these complexes, interactors of well characterized and conserved proteins of the EB1 and XMAP215 family of MAPs were searched for in the model organism *Dictyostelium discoideum*. DdEB1 and the *Dictyostelium* member of the XMAP215 protein family, DdCP224, are known to be part of complexes at the microtubule tips as well as at the centrosome. DdCP224 is involved in centrosome duplication and cytokinesis, whereas DdEB1 assists in spindle formation. At the microtubule tip these two proteins are part of a complex that is thought to link microtubules to the cell cortex.

In this study, two approaches were selected screen for novel interactors.

Employment of the yeast two hybrid system yielded five putative interactors of DdEB1 and DdCP224 that could not be verified by other means.

Tandem affinity purification (TAP) is a method originally established in yeast to isolate highly purified protein complexes in a very gentle and efficient way. In this study TAP was modified for *Dictyostelium* applications and proved to be a useful method to specifically isolate and identify microtubule-associated protein (MAP) complexes. Employing TAP and mass spectrometry the interaction between DdEB1 and DdCP224 was confirmed. Additionally, among several interactions that remain to be confirmed by other methods, an interaction between DdCP224 and a TACC-family protein could be shown for the first time in *Dictyostelium* and was confirmed by colocalization and co-immunoprecipitation analyses. Similar to findings in other species, the TACC domain is sufficient for the centrosomal localization of the protein and the interaction with the XMAP215 orthologue. Based on the results presented, DdTACC, the only member of this protein family present in *Dictyostelium discoideum*, was considered to be an essential gene product.

Zusammenfassung

Das Microtubuli Zytoskelett mit seinen dynamischen Enden ist für viele Funktionen in der Zelle während des gesamten Zellzykluses unerlässlich. Dabei interagieren bekanntermaßen microtubuli-assoziierte Proteine (MAPs) mit anderen Proteinen um all die komplexen Aufgaben rund um die dynamische Instabilität der Mikrotubuli, die Verankerung der Mikrotubuli am Zellcortex, die Kontrolle der Mitose am Centrosom und die Leitung des Transports entlang der Mikrotubuli zu gewährleisten. Fehlfunktionen dieser Proteine führen zu gravierenden Defekten einschließlich Krebs. Die Kenntnis der an diesen Komplexen beteiligen Proteine macht hierbei einen Großteil unseres Verständnisses dieser Prozesse aus.

Um bisher unbekannte Proteine, die zu diesen Komplexen gehören, zu identifizieren, wurde im Modelorganismus *Dictyostelium discoideum* nach Interaktoren von gut charakterisierten und konservierten Proteinen gesucht. Von DdEB1 und DdCP224, dem Vertreter der XMAP215 Proteine in *Dictyostelium discoideum*, ist bekannt, dass sie sowohl Teil des Komplexes an den Mikrotubuli-Plus-Enden als auch desjenigen am Centrosom sind. DdCP224 spielt bei der Centrosomenduplikation und der Zytokinese eine Rolle, DdEB1 hingegen ist an der Bildung der Mitosespindel beteiligt. An den Plus-Enden der Mikrotubuli sind diese beiden Proteine Teil des Komplexes, der die Mikrotubuli vermutlich mit dem Zellcortex verbindet.

In dieser Arbeit wurden nun zwei Ansätze ausgewählt, um die Suche nach neuen Interaktoren aufzunehmen.

Mit Hilfe des Hefe-2-Hybrid Systems wurden fünf mögliche Interaktoren von DdEB1 und DdCP224 entdeckt, die nicht mit andere Methoden bestätigt werden konnten.

Die sogenannte „Tandem affinity Purification“ (TAP), die ursprünglich in Hefe entwickelt wurde, stellt eine sehr sanfte und effiziente Methode dar, nach Proteininteraktionen zu suchen. Sie wurde in dieser Arbeit zur Nutzung in *Dictyostelium discoideum* angepasst und stellte sich als nützliche Methode zur spezifischen Isolation und Identifizierung Mikrotubuli assozierter Proteinkomplexe heraus. Mit Hilfe der TAP und den Methoden der Massenspektrometrie konnte im Rahmen dieser Arbeit die Interaktion zwischen DdEB1 und DdCP224 erneut bestätigt werden. Zusätzlich fanden sich weitere Interaktoren, von denen die zwischen einem Protein der TACC-Familie und DdCP224 erstmalig in *Dictyostelium discoideum* gezeigt werden konnte. Diese Interaktion wurde durch die

Kolokalisierung und Koimmunprezipitation der beiden Proteine bestätigt. Ähnlich wie in anderen Organismen genügt auch in *Dictyostelium discoideum* die TACC-Domäne für die Lokalisation des Proteins am Centrosom und die Interaktion mit dem XMAP215 Orthologen. DdTACC stellt das einzige Mitglied dieser Proteinfamilie in *Dictyostelium discoideum* dar und scheint, wie die Ergebnisse dieser Arbeit nahelegen, essentielle Aufgaben zu besitzen.

I Introduction

1.1 *Dictyostelium discoideum* as a model organism

The natural habitat of the eukaryotic microorganism *Dictyostelium discoideum* is the deciduous forest soil, where it feeds on bacteria and multiplies by equal mitotic division (*Figure 1*). From an evolutionary point of view, *Dictyostelium discoideum* branched off after the divergence of plants (Eichinger *et al.*, 2005), but before the development of metazoans and fungi.

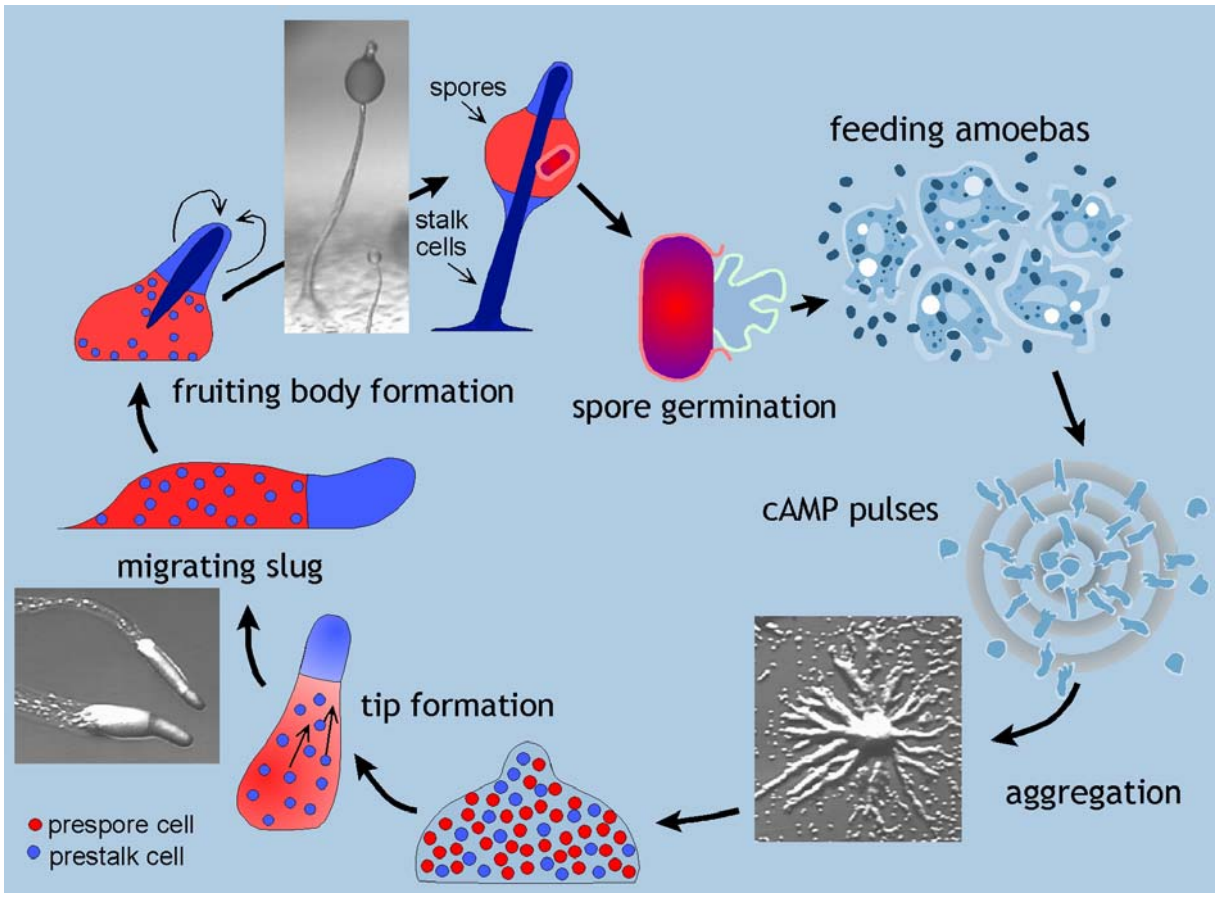

Figure 1 Lifecycle of *Dictyostelium discoideum*. During growth phase *Dictyostelium* exists as single cell amoeba. Upon starvation *Dictyostelium* undergoes chemotaxis towards a pulsatile cAMP wave yielded by the cells emanating from the aggregation centre to its periphery. More than 100,000 cells stream to the centre where they form the mound, which is the first stage in the multicellular development. It forms a tip, which coordinates further development. Next a finger like structure emerges which either immediately culminates into a fruiting body or a motile slug. The slug migrates to a favorable environment before transforming into the fruiting body which is composed of a mass of spores (red cells) supported by a stalk (blue cells). Under suitable conditions spores will hatch out and yield amoebae again, thus closing the cycle. Under standard laboratory conditions fruiting bodies form within 24 h (diagram is taken from the homepage of P. Schaap´s group, University of Dundee).

Upon starvation, the amoebae start to emit pulses of a chemoattractant, cAMP, that induces surrounding cells to move in their direction and to secrete a cAMP pulse themselves and an extraordinary developmental stage of their lifecycle begins (*Figure 1*):

More than 100,000 free-living cells aggregate by chemotaxis towards cAMP to form a multicellular structure. Differentiation into spore and stalk cells takes place in the multicellular structure. This highly complex process is very intriguing from an evolutionary point of view, since about one third of the cells sacrifice themselves as stalk cells for the survival of the rest of the population (Alvarez-Curto *et al.*, 2006; Fortunato *et al.*, 2003) and gave rise to calling *Dictyostelium* strains the social amoebae.

D. discoideum is easy to cultivate and amenable to a variety of biochemical, molecular genetic and cell biological techniques. The molecular genetic techniques available in this context include gene inactivation by homologous recombination, gene replacement, restriction enzyme-mediated integration (REMI), library complementation and expression of multiple fluorescent protein fusion proteins. Since the organism is haploid, mutants can be obtained immediately by homologous recombination. The genome consists of 6 chromosomes with sizes ranging from 4 to 8 Mb (Cox & Mirkin, 1997; Kuspa & Loomis, 1996) which results in a combined total of about 34 Mb of DNA. Including the multicopy 90 kb extrachromosomal element that harbors the rRNA genes, and the 55 kb mitochondrial genome (Eichinger *et al.*, 2005) the estimated number of genes is about 12,500, which makes it about 100 times smaller than mammalian cells. Another important contribution to support the state of *Dictyostelium discoideum* as a powerful model organism is the recently completed genome and cDNA sequencing projects (Eichinger *et al.*, 2005; Morio *et al.*, 1998), which greatly facilitates the performance of proteomic studies. The vibrant community of researchers working with one of the model organisms chosen by the National Institutes of Health as part of its model organism initiative has a very fruitful resource in the website www.dictybase.org. Despite their apparent simplicity, many of the known genes show a high degree of sequence similarity to homologues invertebrate species (Eichinger *et al.*, 2005). *Dictyostelium* amoebae are equipped with a complex actin cytoskeleton that endows the cells with motile behavior comparable to that of leukocytes (Noegel & Schleicher, 2000). Today, 33 orthologues to human genes crucial for a wide range of diseases are identified, making the

organism a great tool towards the aim of understanding disease on a molecular level (Williams *et al.*, 2006). All in all, *Dictyostelium discoideum* offers the advantages of a simple organism that is easy to cultivate while possessing a set of genes much closer related to higher eukaryotes than those of other model organisms such as *S. cerevisiae.*

1.2 The microtubule cytoskeleton

Microtubules form a dynamic network throughout the cell and are required for many essential functions such as cell migration, organelle positioning and mitosis, which make them important targets for anticancer drugs (Wilson & Jordan, 2004).

Structurally, they are hollow tubes with an external diameter of 25 nm, built by systematic polymerization of α - and β - tubulin heterodimers which bind head to tail into protofilaments, while about 13 protofilaments associate in parallel and give rise to a polar cylindrical polymer (Nogales & Wang, 2006). Microtubules can switch stochastically between growing and shrinking phases, a phenomenon known as dynamic instability (Mitchison & Kirschner, 1984). This dynamic character is essential to microtubule function, as evidenced by the large number of Microtubule associated proteins (MAPs) that bind tubulin, alter microtubule dynamics and result in mitotic arrest (Walczak, 2000).

Many MAPs are associated with microtubule plus and minus ends, where they form large protein complexes. The microtubule plus-end complex has a size of more than 2-3 MDa (Karki & Holzbaur, 1999) and mediates the interaction between the cytoskeleton and the cell cortex. The microtubule minus ends emanate from the centrosome, which is the largest protein complex in a eukaryotic cell consisting of possibly over hundred different protein components (Andersen *et al.*, 2003). Their identification has been subject of several studies, e. g. in humans, yeast, *Chlamydomonas*, *Drosophila* and *Dictyostelium* (Andersen *et al.*, 2003; Keller *et al.*, 2005; Lange *et al.*, 2000; Li *et al.*, 2004; Pazour *et al.*, 2005; Reinders *et al.*, 2005; Wigge *et al.*, 1998).

In *Dictyostelium discoideum*, the interphase microtubule system consists of about 30-70 microtubules emanating from the centrosome in a radial fashion towards the cell cortex without much bundling and only little plus- end dynamics but a high lateral motility (Fukui, 1987; Kimble *et al.*, 2000; Koonce & Khodjakov, 2002; Neujahr *et al.*, 1998), In *Dictyostelium*, microtubules are required for organelle transport (Ma *et al.*,

2001; Roos, 1987), but are not essential for cell motility, which seems to be mediated mostly by the actin cytoskeleton (Diez *et al.*, 2005).

During mitosis in *Dictyostelium,* the radially arranged interphase microtubules are disintegrating quickly after release from the centrosome (Fukui, 1987). The mitotic spindle is made up out of overlapping microtubules in between the duplicated centrosomes (Moens, 1976). The nuclear envelope remains intact, but becomes porous so that tubulin dimers can enter through “fenestrae” (McIntosh, 1985; Ueda *et al.*, 1999). The number of spindle-associated microtubules rises to approximately 140-160 at metaphase and early anaphase and the six chromosomes attach to the spindle microtubules at the kinetochores (McIntosh, 1985). During telophase, the spindle elongates to about 3 times of its previous length. Also, a significant number of astral microtubules, which build up the interphase cytoskeleton after cytokinesis arise at this time.

1.3 The centrosome

The centrosome or microtubule organizing center (MTOC) plays an important role during many cellular processes. First of all, it is the site of microtubule nucleation and therefore involved in regulation of the interphase microtubule cytoskeleton. Second, after its duplication it is crucial for the building of the mitotic spindle and for proper cytokinesis and passage from G1/S-phase (Hinchcliffe & Sluder, 2001, Khodjakov & Rieder, 2001; Piel *et al.*, 2001). The presence of supernumerary centrosomes is a hallmark of tumor cells (Lingle *et al.*, 2002; Nigg, 2002).

Despite these ubiquitously essential functions, the morphology of the MTOCs is quite diverse in different cell types and most eukaryotic cells, apart from higher plant cells and female meiotic cells, possess such a structure.

For example the centriolar centrosome of mammalian cells consists of a pair of barrel-shaped centrioles surrounded by a cloud of pericentriolar material (PCM) which is the actual site of microtubule nucleation and anchors the microtubules (Kellogg, 1994) (*Figure 2 A*). Quite different from that is the MTOC of yeast cells called spindle pole body (SPB). It is an acentriolar, disc-like trilaminar structure embedded in the nuclear envelope (Winsor & Schiebel, 1997) (*Figure 2 B*), where the nuclear microtubules emanate from the inner and the cytoplasmic microtubules from the outer of the three layers.

The *Dictyostelium* centrosome structure (shown in *Figure 2 C)* is again different from the mammalian and the yeast MTOCs. Like the yeast spindle pole body, it also lacks centrioles but exhibits a compact layered structure. Similar to mammalian centrosomes, this structure is surrounded by an electron-dense, amorphous matrix that is functionally homologues to the PCM of higher cells. It resides in the cytoplasm during interphase where it is tightly connected to the nucleus by a fibrous linkage (Omura & Fukui, 1985). During mitosis, it inserts itself into an opening in the nuclear envelope.

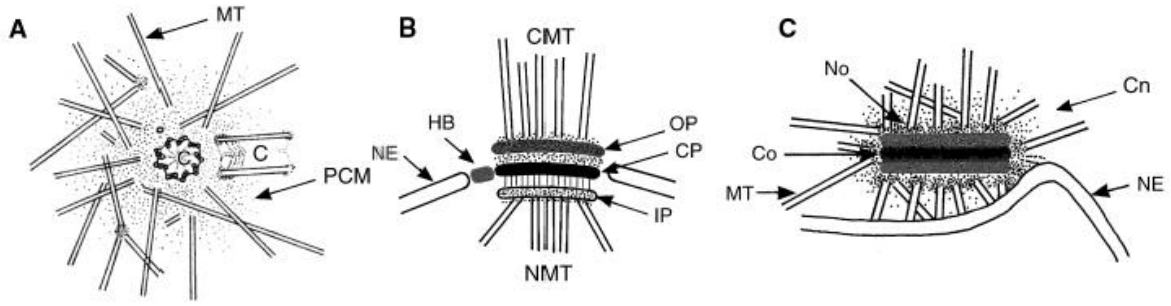

Figure 2 MTOCs in different organisms. A The typical mammalian centrosome with two barrel shaped centrioles (C), which are oriented perpendicular to each other. Microtubules (MT) emanate from the amorphous pericentriolar material (PCM) clustering around the centrioles. **B** The *Saccharomyces cerevisiae* spindle pole body consists of three main layers, the outer plaque (OP) facing the cytosol, the inner plaque (IP) facing the nucleus and the central plaque (CP) which is embedded in the nuclear envelope (NE). Cytoplasmic microtubules (CMT) emanate from the outer and nuclear microtubules (NMT) from the inner plaque. A small structure, called half-bridge (HB) is found next to the inner plaque, which is thought to be the precursor of the duplicating spindle pole body. **C** The box-shaped *Dictyostelium* centrosome consists of a three-layered core structure (Co), which is surrounded by an amorphous corona (Cn). Electron-dense nodules (No) are found in the corona from which microtubules (MT) radiate. The centrosome is linked to the nuclear envelope (NE) via a strong, fibrous linkage, but is not embedded in the membrane. Taken from Daunderer and Gräf, 1999.

The box shaped core structure of the *Dictyostelium discoideum* centrosome is made up of three major layers and surrounded by a corona, which is composed of regularly spaced, dense nodules embedded in an amorphous matrix (Moens, 1976; Roos, 1987). These nodules all contain γ-tubulin and each interphase microtubule appears to emanate from a single nodule (Euteneuer *et al.*, 1998). In *Dictyostelium*, centrosome duplication occurs during mitosis at G2/M-phase and not at G1/S-phase, which is absent in its lifecycle (Weeks & Weijer, 1994). In prophase, the three layered core is enlarged and later the corona dissociates and the interphase microtubules are lost (*Figure 3*). At the transition to prometaphase, the central layer disappears and the two outer layers peel apart to become the mitotic centrosomes. The spindle microtubules then are nucleated from the former inner surfaces of the two layers and start to separate the two mitotic centrosomes. During this process the layers bend

away from the nucleus and eventually, at telophase, they fold back onto themselves and the daughter centrosomes are ready with the microtubules emanating from the former inner surface of the old centrosome's layers (Ueda *et al.*, 1999).

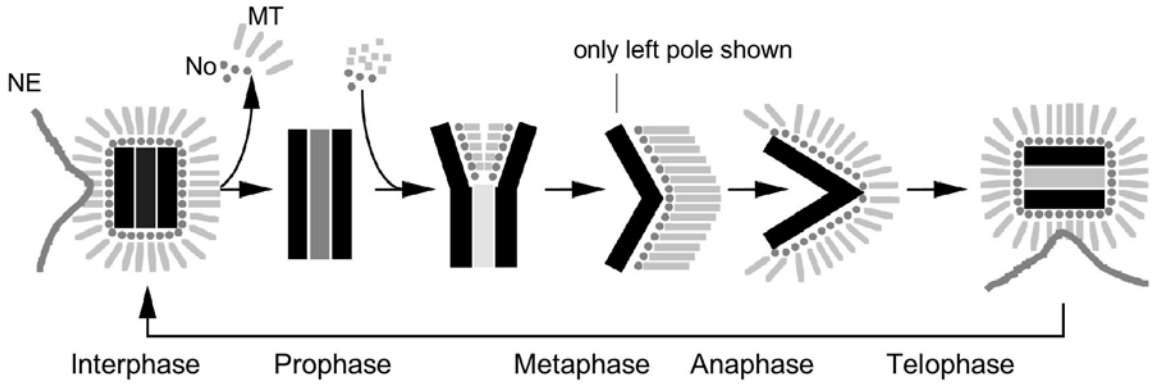

Figure 3 Centrosome cycle in *Dictyostelium discoideum*. The multi-layered core structure of an interphase centrosome is surrounded by an amorphous corona containing dense nodules (No) from which microtubules (MT) emanate. It is associated with the nuclear envelope (NE). At the onset of prophase the core structure increases in size and the corona dissociates. At the onset of prometaphase, the outer layers separate and γ-tubulin (dots) is redistributed to their inner surfaces, forming new nucleation sites for spindle microtubules. The daughter centrosomes then start to fold up and assemble new inner layers, finally resulting in two complete interphase centrosomes. Taken from Gräf, 2000.

Despite the differences in centrosome structure, a lot of the essential proteins are conserved among many species so that most of the mammalian proteins can be found in *Dictyostelium* genome as well (Gräf *et al.*, 2005) while often being highly aberrant in S*. cerevisiae* (Daunderer *et al.*, 1999). Therefore, *Dictyostelium discoideum* is a very suitable model organism to study the composition of the centrosome and the properties of its associated proteins and already has been widely used to do so (Reinders *et al.*, 2005; Schulz *et al.*, 2006).

1.4 Microtubule plus end protein complex

At the opposite end of microtubules, the microtubule plus end, also a variety of important functions needs to be maintained by microtubules and associated proteins. At the cell cortex, the dynamic microtubule plus ends are involved in nuclear migration, spindle orientation, centrosome positioning and directional cell movement (Akhmanova & Hoogenraad, 2005; Euteneuer & Schliwa, 1992; Honnappa *et al.*, 2006; Koonce *et al.*, 1999; Lansbergen & Akhmanova, 2006).

To fulfill these functions, the microtubule plus ends are surrounded by a protein complex (Schuyler & Pellman, 2001). Even though the exact composition of this complex remains unknown, quite a number of proteins present in these fascinating

complexes and their role in the cell have been investigated in the last decade. An important part of it is the dynein/dynactin complex which is made up out of several dynein subunits like the dynein intermediate chain (Beach & Bloom, 2001; Vaughan *et al.*, 1999), p150 glued (Habermann *et al.*, 2001; Vaughan *et al.*, 1999), Arp1, p50 (dynamitin) and p62 (Garces *et al.*, 1999; Valetti *et al.*, 1999; Vaughan *et al.*, 1999) and already has a size of 2-3 kDa (Karki & Holzbaur, 1999). Additionally, proteins like CLIP-170 (Perez *et al.*, 1999) and associated proteins (CLASPs) (Akhmanova *et al.*, 2001), LIS-1 (Coquelle *et al.*, 2002; Rehberg *et al.*, 2005; Schulz *et al.*, 2006), XMAP215, EB1 and the Adenomatous-Polyposis-Coli-Protein (APC) (Mimori-Kiyosue *et al.*, 2000) are known to be part of the microtubule plus end complex. A link between the microtubule and the actin cytoskeleton at the cell cortex seems to be mediated by α- and β-Catenin (Ligon *et al.*, 2001). The overall complex is highly dynamic and its composition is dependent upon the cell cycle, cell type, microtubule dynamics and the interaction with the cortex (Hestermann & Gräf, 2004; Rehberg & Gräf, 2002).

1.5 MAPs

All proteins associated with microtubules form the group of microtubule associated proteins (MAPs) (reviewed in Amos & Schlieper, 2005). Many MAPs are associated with microtubule ends, where they are members of large protein complexes as mentioned above.

Recently, two MAPs, DdEB1 and DdCP224, which are associated with both microtubule plus ends and the centrosome have been characterized in our lab (Gräf *et al.*, 2000; Gräf *et al.*, 2003; Rehberg & Gräf, 2002). They are the *Dictyostelium* representatives of the EB1 and XMAP215-protein families, which comprise the two most universal families of microtubule-associated proteins, since they are present not only in fungi and animals but also in plants. In *Dictyostelium*, both MAPs have already been shown to interact with each other in cytosolic complexes (Hestermann & Gräf, 2004).

1.5.1 EB1

EB1 was first detected as an interactor of the human tumor suppressor protein APC (Su *et al.*, 1995). This interaction is disturbed in most colorectal cancers, which might

explain the chromosomal instability usually associated with cancer of the colon that could be caused by the loss of the connection between microtubules and kinetochores that is mediated by APC/EB1 (Fodde *et al.*, 2001). The name EB1 (end binding protein) reflects part of its localization at the ends of astral microtubules, the kinetochores and the centrosome (Mimori-Kiyosue *et al.*, 2000; Pellman, 2001; Tirnauer & Bierer, 2000). Together with the XMAP215 protein family, the EB1 protein family represents the only MAPs, which are present in all eukaryotic organisms investigated so far. Homologues of human EB1 were characterized in *S. cerevisiae* (Bim1p) (Schwartz *et al.*, 1997), *S. pombe* (Mal3) (Beinhauer *et al.*, 1997), *Drosophila* (DmEB1) (Lu *et al.*, 2001; Rogers *et al.*, 2002) and also in *Arabidopsis* (AtEB1) (Chan *et al.*, 2003).

In addition to its functions at the kinetochores and centrosomes, EB1 is present at the microtubule plus ends, where it mediates cortical anchoring of microtubules. This is demonstrated by its function in spindle orientation and symmetrical division of epithelial cells (Lu *et al.*, 2001) as well as the deletion phenotypes in yeast that show defects in positioning of spindles and nuclei (Beinhauer *et al.*, 1997; Schwartz *et al.*, 1997; Tirnauer *et al.*, 1999).

Another function of EB1 is the enhancement of the dynamic instability of microtubules. The *S. cerevisiae* Bim1p increases the rate of depolymerisation while enhanceing the overall polymerization by an increase in growth time and rescue frequency (Tirnauer *et al.*, 1999; Tirnauer & Bierer, 2000; Tirnauer *et al.*, 2002). EB1 is also widely used as a microtubule tip marker and involved in transport along microtubules (Vaughan, 2005).

Dictyostelium EB1 (DdEB1) has also been thoroughly characterized (Rehberg & Gräf, 2002). Unlike other EB1-family proteins it does not play a major role in the interactions of growing microtubule ends with docking sites at the cell cortex, but similar to its orthologue in *Drosophila* (Rogers *et al.*, 2002) it plays an important role in mitotic progression, i.e. the initiation of spindle formation.

1.5.2 XMAP215 proteins and their *Dictyostelium discoideum* member DdCP224

XMAP215, found as a microtubule stabilizing agent in *Xenopus* egg extracts (Gard & Kirschner, 1987), was the first member of this ubiquitous protein family identified and hence is the name giver for the entire family.

Homologues of *the Xenopus* protein have been found in all eukaryotes investigated thus far including *S. cerevisiae* (Stu 2) (Wang & Huffaker, 1997), *S. pombe* (Dis1/Alp14) (Garcia *et al.*, 2001; Nabeshima *et al.*, 1995), humans (Tog) (Charrasse *et al.*, 1998), *Drosophila* minispindles (Cullen *et al.*, 1999), C. elegans (Zyg9) (Kemphues *et al.*, 1986) and *Arabidopsis* (Mor1) (Whittington *et al.*, 2001) suggesting general and indispensable functions (Ohkura *et al.*, 2001).

Intriguingly, in addition to their microtubule stabilizing activity, XMAP215 proteins also exhibit destabilizing activity in the yeast homologue stu2 and under specific conditions also in *Xenopus* (Shirasu-Hiza *et al.*, 2003; Van Breugel *et al.*, 2003). Studies of the *Drosophila* homologue minispindles suggest that the influence of XMAP215 proteins on microtubule dynamics results from a regulation of pauses, since the depletion leads to an increased paused state of microtubules (Brittle & Ohkura, 2005).

In addition to their influence on microtubule plus end dynamics, XMAP215 proteins also fulfill important functions at the microtubule minus ends, i.e. centrosomes, spindle pole bodies and microtubule organizing centers (MTOCs). Here, XMAP215 proteins are involved in microtubule nucleation and centrosome duplication (Gräf *et al.*, 2003; Popov *et al.*, 2002; Popov *et al.*, 2001).

The *Dictyostelium* member of this protein family, DdCP224, was detected by generation of monoclonal antibodies against isolated centrosomes (Gräf *et al.*, 1998), which allowed identification of DdCP224 as a centrosomal component (Gräf *et al.*, 1999). DdCP224 has since been studied by our lab and we now know that it is involved in centrosome duplication, cytokinesis, microtubule growth and microtubule plus end/cell cortex interactions (Gräf *et al.*, 2000; Gräf *et al.*, 2003; Hestermann & Gräf, 2004). The C-terminal 460 amino acids of DdCP224 are sufficient for centrosomal binding (Hestermann *et al.*, 2002), whereas a construct consisting of the N-terminal 813 amino acids localizes to the cell cortex (Hestermann & Gräf, 2004).

1.6 Aims of this study

To gain insights about the composition of the protein complexes at either ends of microtubules is crucial for our understanding of the cytoskeleton and its important functions in health and disease. Therefore, the aim of this study was to search for interactors of two well conserved known members of these complexes, which are present in all eukaryotes studied so far, EB1 and the XMAP215 family protein DdCP224.

The yeast two hybrid system is a well established tool to detect protein-protein interactions and screen genomic libraries. Since a *Dictyostelium discoideum* cDNA library was readily available to our lab, this method was chosen as a first approach in search for DdCP224 and DdEB1 interactors.

As the complete sequence of *Dictyostelium discoideum* became available during the course of this study and made proteomic approaches feasible (Eichinger *et al.*, 2005), another method relying on the detection of proteins by mass spectrometry seemed to be a promising technique in search for DdCP224 and DdEB1 interactors:

Tandem affinity purification was first developed in yeast (Puig *et al.*, 2001; Rigaut *et al.*, 1999) and has been proven to be a valuable tool to screen for protein interactions in this organism (Shevchenko *et al.*, 2002).

In the course of this study this method should be adapted for the use with *Dictyostelium discoideum* to combine the advantages of the method with the possibility to look for interactions directly in the organism of interest. Tandem affinity purification should provide another valuable tool for the model organism *Dictyostelium discoideum* and more information on microtubule associated proteins.

II Materials and Methods

1 Materials

1.1 Reagents

Unless stated otherwise chemicals were obtained from Biorad (München), Fluka (Buchs, Switzerland), Merck (Braunschweig), Roche (Mannheim), Carl Roth (Karlsruhe), Serva (Heidelberg), Sigma-Aldrich (Deisenhofen), Difco (Augsburg), AppliChem (Darmstadt) and Boehringer Mannheim (Mannheim) and were of p. a. quality. Other materials were supplied mainly by Greiner (Frickenhausen), Nunc (Wiesbaden), Qiagen (Hilden), Macherey-Nagel (Düren) and Sarstedt (Nümbrecht).

1.2 Antibodies

Antibodies against DdEB1, the N-terminal part (anti-DdCP-HindIII) and C-terminal part of DdCP224 (anti-DdCP224 mAb 2/165) and GFP were described previously (Faix *et al.*, 2001; Gräf *et al.*, 1999; Hestermann & Gräf, 2004; Rehberg & Gräf, 2002). Additionally, a commercially available polyclonal antibody, which recognizes part of the calmodulin binding peptide up to the TEV cleavage site (Anti-TAP antibody; BioCat, Heidelberg, Germany) was used for immunofluorescence microscopy.

Anti-Comitin,	mAb	(Weiner *et al.*, 1993)
Anti-DdEB1,	Rabbit Antiserum	(Rehberg & Gräf, 2002)
Anti-DdCP224,	DdCP224HIND Rabbit Antiserum	(Hestermann & Gräf, 2004)
Anti-DdCP224,	mAb 4-148	(Gräf *et al.*, 1999)
Anti-γ-tubulin,	Rabbit Antiserum	(Euteneuer *et al.*, 1998)
Anti-GFP,	Rabbit Antiserum	(Faix *et al.*, 2001)
Anti-GFP,	mAb 264-449-2	(Weber, 1999)
Anti-GFP,	mAb 264-236-1	(Chemicon, Hofheim)
Anti-MBP,	Rabbit Antiserum	(Gräf, 2001b)
Anti-DdTACC,	TACC domain Rabbit Antiserum	(this study)

Anti-Tubulin, YL1/2	Chemicon, Hofheim
Anti-Digoxigenin	Boehringer Mannheim
Goat-anti-Rabbit IgG Antibody, coupled to alcaline phosphatase	Sigma
Goat-anti-Mouse IgG Antibody, coupled to alkaline phosphatase	Sigma
Goat-anti-Rabbit IgG Antibody, bound to Cy3 or FITC	Dianova
Goat-anti-Mouse IgG Antibody, bound to Cy3 or FITC	Dianova
Goat-anti-Rabbit Alexa 488 or 568	Molecular probes
Goat-anti-Mouse Alexa 488 or 568	Molecular probes
Goat-anti-Rat Alexa 488 or 568	Molecular probes

1.3 Enzymes

DNA modifying enzymes from New England Biolabs (Frankfurt) were used in this study unless stated otherwise.

1.4 Antibiotics

Blasticidin S	MP Biochemicals
Geneticin (G418)	GIBCO BRL
Penicillin/Streptomycin	Sigma
Ampicillin	Roth
Kanamycin	Sigma

1.5 Buffers and solutions

Buffers and solutions not listed below are described together with the method they have been used for.

Soerensen buffer (Malchow *et al.*, 1972)
14.6 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6.0.

PHEM-buffer (Schliwa, 1982)
60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9.

10 x TE-buffer
10 mM Tris/HCl, 1 mM EDTA, pH 8.0.

20 x SSC
3 M NaCl, 0.3 M Na-Citrate, pH 7.0.

10 x TAE
400 mM Tris, 10 % acetic acid, 10 mM EDTA, pH 8.3.

10 x PBS
70 mM Na_2HPO_4, 30 mM KH_2PO_4, 150 mM NaCl, pH 7.4.

10 x TBS
200 mM Tris/HCl, pH 7.2, 1.5 M NaCl.

20 x TBST
20 mM Tris/HCl, pH 7.2, 150 mM NaCl, 0.05 % Tween-200.

Urea sample solution
9 M urea, 10 % SDS, 5 % 2-mercaptoethanol.

5 x Laemmli sample buffer
625 mM Tris/HCl, pH 6.8, 25 % sucrose, 10 % SDS, 0.025 % bromphenolblue, 10 %, 2-mercaptoethanol.

SDS sample buffer
625 mM Tris/HCl, pH 6.8, 10 % SDS, 0.025 % bromphenolblue, 100 mM DTT, 1 drop glycerol.

10 x SDS running buffer
1 M Tris/HCl, pH 8.3, 1 % SDS (w/v), 1 M Glycin.

1.6 Software

Word X, Excel and PowerPoint (Microsoft Office X), Adobe Photoshop 7.0, Endnote 8.0, Image J 1.34l, NIH-Image 1.6.2, DNA Strider 1.4f6 (all Macintosh), Zeiss LSM 510Meta Software 3.2, Heugens Essential 2.4.1.

1.7 Other materials

NHS-Sepharose 4B	GE Healthcare
Hybond N Nylon membrane	GE Healthcare
Nitrocellulose BA85	Schleicher & Schüll
Membrane for dialysis	Biomol

2 Organisms and microbiological methods

2.1 Organisms

2.1.1 *Dictyostelium* strains

D. discoideum strain AX2-214 (axenic growing derivate of isolate NC-1 (Raper, 1935)) was used in this study. Other strains used in this study (generated from AX2) are

D. discoideum strain ΔEB1	(Rehberg & Gräf, 2002)
D. discoideum strain GFP-DdCP224ΔC	(Hestermann & Gräf, 2004)

Strains generated in this study all derive from plasmids listed in *Table 2* and are described in 3.18 (Generation of Constructs).

2.1.2 Bacterial Strains

Escherichia coli strains DH5α (Sambrook *et al.*, 1989), Rosetta (Novagen) and XL1-Blue (Stratagene) were used for cloning.
Klebsiella aerogenes (Williams & Newell, 1976) was used for cultivation of *D. discoideum*.

2.1.3 *S. cerevisiae* strains

Strains used in this study were obtained from Clontech.

AH109 MATa, trp1-901. leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3,$GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ

Y187 MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met^-, gal80Δ, URA3::$GAL1_{UAS}$-$GAL1_{TATA}$-lacZ

2.2 Cultivation and preservation of organisms

2.2.1 Media and cultivation of *D. discoideum*

Dictyostelium cells were cultured axenically in AX-medium containing Blasticidin S or G418 in case of mutants with 150 rpm on a rotary shaker at 21°C. Under these conditions doubling time was about 8 h. Backup cultures of adherent cells were kept in HL5c medium in small tissue culture flasks and medium was changed twice a week.

For long term storage cells were subjected to starving conditions, inducing the formation of spores, which can easily be frozen and stored. For this, axenically growing cells were washed twice with Soerensen buffer, resuspended at a density of 2-3 x 10^8 cells/ml and 500 µl of the suspension were plated out on freshly prepared phosphate agar plates. Cells formed spores containing fruiting bodies within 2-3 days, which were washed off with sterile Soerensen buffer (about 5 ml per plate), shock-frozen in 1 ml aliquots (Nunc 2.2 ml tubes) in liquid nitrogen and stored at -70°C. For inoculation of a shaking culture, spores were thawed at room temperature and resuspended in 30 ml HL5c medium. After 3 days the cultures usually had a density of about 5 x 10^6 cells/ml.

AX Medium (Claviez *et al.*, 1982)

14.3 g/l peptone (Oxoid), 7.15 g/l yeast extract (Oxoid), 18 g/l glucose, 0.5 g/l Na_2HPO_4, 0.45 g/l KH_2PO_4, pH 6.7.

HL-5c Medium

5 g/l yeast extract (Difco), 2.5 g/l bacto tryptone (Difco), 2.5 g/l casein peptone (Merck), 5 g/l proteose peptone (Oxoid) 10 g/l glucose, 1.2 g/l KH_2PO_4, 0.35 g/l Na_2HPO_4, pH 6.5.

Phosphate solid medium

15 g/l bacto agar in Soerensen buffer.

SM solid medium

10 g/l peptone (Oxoid), 1 g/l yeast extract (Oxoid), 10 g/l glucose, 20 g/l bacto agar, 1 g/l K_2HPO_4, 2.2 g/l KH_2PO_4, 1 g/l $MgSO_4$, pH 6.5.

2.2.2 Media and Cultivation of *E. coli*

E. coli cells were grown according to standard methods (Sambrook *et al.*, 1989) on agar plates or shaking at 240 rpm at 37°C. For protein expression the temperature was reduced to 22°C.

For long-term storage at –70°C, cultures were supplemented with 35 % sterile glycerol.

LB Medium

10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0.

For solid medium, 1,5 % agar was added. If necessary, ampicillin or kanamycin were added from stock solutions to a final concentration of 100 µg/ml.

SOB Medium

20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.55 mM KCl.

2.2.3 Yeast media and cultivation

S. cerevisiae strains were cultured according to standard procedures (Guthrie & Fink, 2002) either in complete (YPD) or Synthetic Complete Drop-out medium supplemented as appropriate with 20 µg/ml adenine, uracil, tryptophan, histidine and methionine or 30 µg/ml leucine and lysine. Strains were grown at 30°C. For long-term storage at –70°C, cultures were supplemented with 50 % sterile glycerol.

YPD

10 g/l yeast extract, 20 g/l peptone, 2 g/l glucose

YPAD medium

6 g/l yeast extract (Difco), 12 g/l Peptone (Difco), 12 g/l Glucose, 60 mg/l adenine hemisulphate

Synthetic Complete Drop-out medium

4 g/l Difco Yeast Nitrogen Base (w/o amino acids), 12 g/l glucose, 0.5 g/l Synthetic Complete Drop Out Mix

For solid media, 10 g/l Difco Bacto Agar was added.

Synthetic Complete drop-out medium mix

2.0 g Adenine hemisulfate
2.0 g Arginine HCl
2.0 g Histidine HCl
2.0 g Isoleucine
2.0 g Leucine
2.0 g Lysine HCl
2.0 g Methionine
3.0 g Phenylalanine
6.0 g Homoserine
3.0 g Tryptophan
2.0 g Tyrosine
1.2 g Uracil
9.0 g Valine

For the preparation of LWH-medium, Histidine, Leucine and Tryptophan were left out of the mixture, LWHA-medium also lacks Adenine. For the galactosidase assay, 1 ml of a X-α-Gal solution (20 mg/ml in dimethylformamide) was added to 1 L medium before pouring the plates.

3 Molecular biology methods

3.1 DNA cleavage with restriction enzymes

Restriction digests were performed using the buffer system and temperature recommended by the manufacturer (New England Biolabs, Frankfurt). Reaction volume was at least 15 µl. 1-5 units enzyme per µg DNA was used. Incubation time was at least 1 hour. Completion of the digests was analyzed on agarose gels (3.2).

3.2 Agarose gel electrophoresis

The separation of DNA fragments according to their size was performed using gels with 1 % to 2 % agarose in TAE buffer. Samples were mixed with 1/5 volume of 6 x DNA loading dye before loading. Gels were run with 5 V/cm. For detection of DNA fragments gels were stained for 20 min in TAE buffer containing 1 µg/ml ethidium bromide. Bands were detected by UV illumination and documented with the Eagle Eye II CCD camera system (Stratagene, Heidelberg).

TAE

40 mM Tris, 0.1 % acetic acid, 1 mM EDTA, ph 8.3

6 x DNA loading dye

10 mM Tris/HCl, pH 8.0, 50 mM Na-EDTA, pH 8.0, 1 % SDS, 30 % glycerol, 0.1 % bromphenole blue

3.3 DNA extraction from agarose gels

DNA bands were excised with a scalpel, transferred to sterile Eppendorf vials, weighed and purified with Qiaquick columns (Qiagen, Hilden) following the instructions of the manufacturer.

3.4 Determination of DNA concentration

DNA concentration in solutions was determined by measuring the extinction at 260 nm (E_{260}) of the diluted sample after calibration of the photometer with a buffer control. An E_{260} of 1.0 corresponds to 50 µg/ml of doublestranded DNA (Sambrook *et al.*, 1989).

3.5 Preparation of plasmid DNA

Plasmid DNA was prepared from overnight cultures using the Qiagen-Plasmid-Kit (Qiagen, Hilden) or the Macherey-Nagel (Düren) kit. For small scale preparations (3 ml) the Qiagen's manual for 'mini-preps' excluding the Tip20-column was followed, for large scale preparations (100-200 ml) the manual for 'midi-preps'.

3.6 Polymerase chain reaction (PCR)

Amplification of DNA fragments was carried out by 'polymerase chain reaction'. Standard PCRs were carried out using Tag-polymerase from various sources. The 25 µl reactions contained 0.8 mM dNTP (0.2 mM of each nucleotide), 25 pmol 5'- and 3'-Primer, 1 U Taq-Polymerase and 2.5 µl 10 x PCR buffer (100 mM Tris/HCl, pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.1 % (w/v) Gelatin). $MgCl_2$ concentration was varied to get more or less stringent conditions. Plasmid or genomic DNA, λphages and cDNA was used as template. Prior to amplification the reaction mix was denatured for 2 min at 94°C (or 5-10 min for genomic DNA and λphage templates). In general, 25-30 cycles were run (denaturation: 30 s at 94°C, annealing: 45 s at temperature according to oligonucleotide hybridizing temperature calculated from base constitution, elongation: length according to product length (1,000 bp per min) at 72°C). The 'Expand High Fidelity Polymerase Mix' (Roche, Penzberg) was used for preparative PCR reactions according to manufacturer's instructions.
The PCR product was isolated from nucleotides and enzyme by the 'Qiaquick PCR Purification Kit' (Qiagen, Hilden).

3.7 Reverse Transcription – PCR (RT-PCR)

This method was used to amplify the full length sequence of genes detected by yeast two hybrid screen. 1 µg polyadenylated RNA were mixed with 1 µg of an oligo dT primer in a total volume of 18 µl, denatured at 70°C for 5 min and immediately immersed in ice water for 3 min. Reverse transcription was initiated by addition of 5 µl 5 x RT buffer, 2 µl reverse transcriptase and carried out for 1 h at 42°C. 2 µl of the reverse transcription reaction were used as template for subsequent amplification by PCR using a specific primer pair.

3.8 Oligonucleotides

Oligonucleotides were purchased from ThermoHybaid (Ulm) and biomers (Ulm). The following oligonucleotides are given from 5' to 3' end.

Name	Sequence
38-27Bam	CGCGGATCCATGAGAAGTATTTTATCTTTATT
38-27ganzFor	ATGAGAAGTATTTTATCTTTATTAATTG
38-27ganzForSal	TACGCGTCGACTAATGAGAAGTATTTTATCTTTATT
38-27ganzRev	TTATTGTACAAAGGATAATGTTGAAATAATTC
38-27ganzRevBam	CGCGGATCCTTATTGTACAAAGGATAATGTTGAAAT
38-27neur	TTAATTTTCACATTTTCCATCTTTCC
38-27neurBam	CGCGGATCCTTAATTTTCACATTTTCCATCTTTCC
38-27rsal	TACGCGTCGACTTTAATTTTCACATTTTCCATC
38-32ganzFor	ATGGTTCATGTATCAAGCTTTAAAAACG
38-32ganzForSal	TACGCGTCGACTAATGGTTCATGTATCAAGCTTTA
38-32ganzRev	TTATAAATCACTACCAAAAGTATTTTCACC
38-32ganzRevBam	CGCGGATCCTTATAAATCACTACCAAAAGTATT
apg8fBam	CGCGGATCCATGGTTCATGTATCAAGCTTTA
apg8rSal	TACGCGTCGACTTTATAAATCACTACCAAAAGTAT
Bsrforw	CTCATTCCACTCAAATATACCCGAAATTAA
Bsrrev	CAGTTACTCGTCCTATATACG
E6fBam	CGCGGATCCATGACAATAACCAATTATCC
E6GANZFOR	ATGACAATAACCAATTATCCATTTG
E6GANZFORSAL	TACGCGTCGACTAATGACAATAACCAATTATCC
E6GANZREV	TTAAAAGATAACACCACGTAATC
E6GANZREVBAM	CGCGGATCCTTAAAAGATAACACCACGTAATC
E6rBam	CGCGGATCCAATGACAATAACCAATTATCC
E6rSal	TACGCGTCGACTTTAAAAGATAACACCACGTAATC
E26GANZFORSal	TACGCGTCGACTAATGGGTAATAAACAAGGTAAATC
E26fBam	CGCGGATCCATGGGTAATAAACAAGGTAAATC
E26GANZFOR	ATGGGTAATAAACAAGGTAAATCC
E26ganzrev	TTAATCAAAAGTAATTGGCACGTC
E26ganzrevBam	CGCGGATCCTTAATCAAAAGTAATTGGC
E26rSal	TACGCGTCGACTTTAATCAAAAGTAATTGGC
E67neur	TTAATTTTTTATTCCCAATGCAAC
E67fBam	CGCGGATCCATGAAGTCAAAACGTTA

Name	Sequence
E67neurbam	TTAATTTTTTATTCCCAATGCAACGGATCCGCG
EB1-67neuf	ATGAAGTCAAAACGATTATTTTTTTTATTATGC
E67rSal	TACGCGTCGACTTTAATTTTTTATTCCCAATGC
EB1-67neufSal	TACGCGTCGACTAATGAAGTCAAAACG
fTAPBam	CGCGGATCCATGGAAAAGAGAAGATGG
rTAPNsi	CGCGGATGCATTCAGGTTGACTTCCCCG
TACC-2	GAATTAATTTTTAAATTACAAACTAATCAAAAA
TACC-2SAL	ACGCGTCGACGTTTTTGATTAGTTTGTAATTTAAAAATTAATTC
TACC-3	TCACAAGATGGATTTAATTTACAATC
TACC-3BamHI	CGCGGATCCTCACAAGATGGATTTAATTTACAATC
TACC3pst	TATATACTGCAGCAAGATGGATTTAATTTACAATC
TACC3Sac	CCGAGCTCGTCACAAGATGGATTTAATTTACAATC
TACC7SalF	TACGCGTCGACAATGGATAATGAAAAATTAAAAAG
TACC8BsgIr	CATCTTCAGGTGTTTTTTGTTTTGCTGCACCTTTTGC
TACC9BsgIf	GCAAAAGGTGCAGCAAAACAAAAAACACCTGAAGATG
TACC10Afl3r	CCCACATGTTTTGATGTGGTGGTGGATAATAAAATTG
TACC11Afl3f	CCCACATGTTTACTCAAGAAGATATTGATCG
TACCko1Kpn	GGGGTACCCCGTCAATTATTGGGTTTATTTGG
TACCko2Hind	CTCTTTAGACAAGCTTCTTTTTAATTTTTCATTATCCAT
TACCKOtest1	GCGGATTTAGAAATTACAAAATCAAACC
TACCKOtest2	CCTGTTGCTAAAGTGGCAATTGC

Table 1 Oligonucleotides used in this study

3.9 Dephosphorylation of DNA

When ligating DNA fragments with compatible ends, the probability of vector religation is very high. To prevent this, vector deposphorylation was carried out with calf intestinal phosphatase (Sambrook *et al.*, 1989) which catalyzes the dephosporylation of 5'-phosphates from DNA and RNA. 2.5 µg linearized vector DNA were incubated in a 25 µl reaction in 1 x CIP buffer (50 mM Tris/HCL, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidin) or NEB buffer 2-4 with 1 U CIP for 30 min at 37°C. the reaction was terminated by heating to 65°C for 10 min and the DNA was subsequently purified on an agarose gel.

3.10 Ligation of DNA into plasmid vectors

Vector and DNA fragments were cleaved (3.1), separated on agarose gels by electrophoresis (3.2), and extracted from agarose gels (3.3). DNA fragments were ligated with T4 DNA ligase (New England Biolabs, Frankfurt) in a volume of 10 µl at 16°C for 2 hours or overnight using the buffer system supplied by the manufacturer. The ratio of vector to insert was about 1:2 for sticky end ligations, the concentration being estimated from band intensities on analytical agarose gels. 2 µl of the reaction was transformed into competent *E. coli* cells (3.11).

3.11 Preparation and transformation of chemically and electrocompetent *E. coli* cells

3.11.1 Preparation of electrocompetent cells

400 ml LB medium was inoculated with 10 ml of an *E. coli* XL1-Blue overnight culture and grown to an OD_{600} of 1 at 37°C under vigorous shaking. All flasks and solutions subsequently used were sterilized and cooled to 4°C. Quality of the competent cells depended on consequent cooling. Cells were harvested by centrifugation (GSA rotor: 4,000 rpm, 15 min, 4°C) and resuspended in 200 ml H_2O. After another centrifugation the cells were resuspended in 100 ml H_2O, pelleted again, washed with 20 ml of 10 % glycerol and finally resuspended in 2 ml of 10 % glycerol. After aliquotting in 100 µl segments the cells were frozen in liquid nitrogen and stored at -70°C.

3.11.2 Electroporation

For transformation, electrocompetent cells were thawed on ice. 100 µl cells were mixed with 0.5 µl vector or 2 µl ligation reaction and placed in a precooled, sterile electroporation cuvette (Eurogentec; distance between electrodes 2 mm). After a pulse (2.5 kV, 25 mF and 200 Ω in an electroporation device (Gene Pulser, Biorad)) 1 ml of SOC medium was added immediately, gently agitated for 30 min at 37°C and plated on LB agar plates with the appropriate antibiotic for selection.

SOC

SOB medium with 10 mM $MgSO_4$, 20 mM $MgCl_2$, 20 mM glucose

3.11.3 Preparation of chemically competent cells

200 ml LB medium was inoculated with 5 ml of an *E. coli* DH5α overnight culture and grown for 2.5 h at 37°C under vigorous shaking. All flasks and solutions subsequently used were sterilised and cooled to 4°C. Quality of the competent cells depended on consequent cooling. Cells were harvested by centrifugation (GSA rotor: 4,000 rpm, 10 min, 4°C) and washed with 0.1 M $CaCl_2$ and 20 % glycerol. The pellet was kept on ice for 1 h and finally resuspended in 12 ml of 0.1 M $CaCl_2$ and 20 % glycerol. After aliquotting in 200 µl the cells were frozen in liquid nitrogen and stored at -70°C.

3.11.4 Heat Shock transformation

For transformation, chemically competent cells were thawed on ice. 200 µl cells were mixed with 35 µl TCM buffer and 0.5 µl vector or 10 µl ligation reaction and incubated on ice for 60 min. The mixture was placed at 42°C for 75 s, then on ice for 10 min. After addition of 400 µl LB medium and 30 min incubation at 37°C, the mixture was plated on LB agar plates with 100 µg/ml ampicillin (DH5α).

TCM buffer

10 mM Tris HCl, pH 7.5, 10 mM $CalCl_2$, 10 mM $MgCl_2$

3.11.5 Identification of transformed clones in *E. coli*

DNA of transformed bacteria was isolated (3.5), cleaved with appropriate restriction endonucleases (3.1) and analyzed on agarose gels (3.2). Plasmids with the expected restriction fragments were sequenced by Delphiseq, Regensburg or Biolux, Stuttgart. Sequences were aligned with DNA Strider.

3.12 Transformation of *S. cerevisiae*

Competent yeast cells were created and transformed as described by Gietz (http://www.umanitoba.ca/faculties/medicine/biochem/gietz/) on the yeast transformation homepage. In this study, high efficiency transformation was used to transform the bait constructs into the strain containing the library plasmids. For all other transformations, the “quick and easy” protocol was used. (Gietz & Woods, 2002)

High efficiency transformation

The yeast strain, which is to be transformed, is inoculated into 5 ml of liquid medium (2 x YPAD or appropriate SC selection medium) and incubated overnight on a rotary shaker at 200 rpm and 30°C. On the next day, 2.5 x 10^8 cells of the overnight culture are added to 50 ml of pre-warmed 2 x YPAD in a pre-warmed culture flask to give 5 x 10^6 cells/ml, which are again incubated until cells have completed a least two divisions and a titer of at least 2 x 10^7 cells/ml is reached. Cells are harvested by centrifugation at 3,000 g for 5 min, washed in 25 ml of sterile water and resuspended in 1 ml of sterile water and washed again. 100 µl of this 1 ml cell suspension are sufficient for one transformation. To each sample of pelleted cells 240 µl PEG 3,500 (50 % w/v), 36 µl LiAc (1.0 M), 50 µl boiled SalmonSperm-carrier DNA and 34 µl Plasmid DNA plus Water are added and mixed by vigorous vortexing. Tubes are incubated in a 42°C water bath for 40 min and pelleted by centrifugation. 1 ml dH_2O is added to the cell pellets and 200 µl each are plated on a SC medium plate.

Quick and easy yeast transformation

Instead of using cells from an overnight culture and letting them grow until they reach a titer of at least 2 x 10^7 cells/ml, a blob of cells is scraped from a freshly grown colony on a plate and suspended in 1 ml of sterile water. The transformation itself is performed as described in the high efficiency transformation protocol.

3.13 Yeast two-hybrid screening

Yeast two-hybrid interactions were analyzed with the Matchmaker two-hybrid system 3 according to the manufacturer's instructions (Clontech/BD Biosciences, Palo Alto, USA).

3.14 Preparation of plasmid DNA from yeast

For preparation of yeast plasmid DNA for subsequent transformation of E. coli, 1.5 ml of an overnight culture (OD_{600} > 1) were pelleted by centrifugation and resuspended by vortexing in 200 µl of SCE/Zymolyase/ßME. The mixture was incubated at 37°C 30 - 60 minutes for cell wall digestion. After complete cell wall digestion, 400 µl 0.2 N NaOH/1 % SDS (made fresh) were added and mixed by inversion. Subsequent to

incubation on ice 5 minutes, 300 µl cold 3 M K/5 M OAc were added and the mixture was incubated on ice 5 minutes.
After centrifugation at 14,000 rpm for 2 min, supernatant was poured into a fresh tube. After a second centrifugation, 500 µl were transferred to a fresh tube and 300 µl isopropanole were added, the mixture was vortexed and let stand at RT for 5 min. After centrifugation at 14,000 rpm for 5 min, supernatant was poured off and the pellet was washed with 0.5 ml 70 % ethanol.
The dried pellet was resuspended in 25 µl TE and 1 µl was used to transform electrocompetent *E. coli* cells, whereas 10 µl were used to transform chemically competent *E. coli* cells (see section 3.11).

SCE Solution
1 M sorbitol, 0.1 M sodium citrate pH 7.6, 0.06 M EDTA
SCE/Zymolyase/ßME Solution
5 ml SCE, 60 µl 10 mg/ml Zymolyase (in 1 M Sorbitol), 10 µl ß-mercaptoethanol
NaOH/SDS Solution (made fresh)
100 µl 10N NaOH, 500 µl 10 % SDS, 4.4 ml dH_2O
3 M K/5 M OAc
60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml dH_2O

3.15 Preparation of chromosomal DNA from *D. discoideum*

1-2 x 10^8 cells of an axenically growing culture were washed twice with cold H_2O and the cell pellet was resuspended in 50 x lysis buffer (10 mM Mg-acetate, 10 mM NaCl, 30 mM HEPES, pH 7.5, 10 % sucrose, 2 % Nonidet P40). Cells lysed upon this treatment and nuclei were sedimented at 600 g (10 min, 4°C), resuspended in SDS-lysis buffer (TE buffer with 0.7 % SDS) and supplemented with 100 µl proteinase K solution (14,7 mg/ml). After 2-3 h incubation at 60°C the lysate was carefully extracted with an equal volume of phenol/chloroform (Sambrook *et al.*, 1989) until the upper phase was clear (2-4 times). DNA was precipitated by addition of 1/10 volume of 2 M Na-acetate (pH 5.2) and 2 volumes of ethanol and the white threads of DNA were fished with a glass hook. DNA was washed in 70 % ethanol, air dried and dissolved in 200-500 µl of TE buffer. Alternatively, DNA was purified with the High Pure PCR Template preparation kit (Roche, Mannheim) according to manufacturer's instructions.

3.16 Transformation and cloning of *D. discoideum*

Electroporation

Dictyostelium cells were grown to a density of 2-3 x 10^6 cells/ml, harvested and washed once in cold Soerensen buffer and twice in cold electroporation buffer (50 mM sucrose, 10 mM KH_2PO_4, PH 6.1). Cells were resuspended in cold electroporation buffer at a final density of 1 x 10^8 cells/ml, mixed with 15-30 µg of plasmid DNA and transferred to a precooled, sterile electroporation cuvette (distance between electrodes 4 mm). After two pulses (1.0 kV, 3 µF) in an electroporation device (Gene pulser, Biorad) cells were transferred to a sterile tissue culture dish for a 15 min recovery period at room temperature. After supplementation with an $MgCl_2/CaCl_2$ solution (final concentration 1 mM each) cells were gently agitated for another 15 min at room temperature. Finally cells were resuspended in 25 ml HL-5c medium and distributed into a 24-well plate in case of Blasticidin resistance being used. After a recovery period of 24 hours, 4 µg/ml Blasticidin S was added and the cells were incubated for 8-14 days until colonies of resistant cells appeared. In case of G418 resistance being used, cells were first incubated in liquid medium for 24 h and then plated on phosphate agar plates together with a dense solution of freshly grown *Klebsiella aerogenes* cells. After 3-14 days incubation at 21°C feeding plaques appeared and transformants were lifted with a sterile pipette tip from the edges and transferred to a 24-well plate with HL-5c medium containing G418 (10 µg/ml) and a Penicillin/Streptomycin solution (Sigma).

Cloning of transformants

Transformants resulting from Blasticidin containing constructs were resuspended with a sterile pipette, a droplet of the cell suspension was transferred to a coverslip and cells were examined by immunofluorescence microscopy (5.1). If cells with the desired label were found, different concentrations of the remaining cells were plated on SM agar plates together with a dense suspension of *Klebsiella aerogenes* cells and proceeded as described for transformants containing G418 resistance, except Blasticidin S was used instead of G418.

3.17 Isolation of polyadenylated RNA from *D. discoideum*

Polyadenylated RNA (mRNA) was prepared with the QuickPrep mRNA micro kit (GE Health Care) according to the instructions of the manufacturer. The yield was consistently 7-8 µg of mRNA per 1 x 10^7 cells and mRNA was precipitated in 2-4 aliquots of 1/10 volume 2 M K-acetate and 2 volumes of ethanol and glycogen and stored at –70°C until use. Precipitated mRNA was recovered by centrifugation at 14,000 rpm (Beckman CS-15R centrifuge, F2402 rotor), washed with 70 % ethanol in DEPC-treated water, air–dried and dissolved in DEPC-treated water.

3.18 Generation of Constructs

A *Dictyostelium* C-terminal TAP tag vector containing a blasticidin resistance cassette was constructed by adding a Bam HI/Nsi I fragment from the yeast TAP tag vector pBS1539 (Puig *et al.*, 2001) to p1ABsr8 (Gräf *et al.*, 2000). For pKK7 (EB1-TAP) full length DdEB1 was cut from GFP-DdEB1 (Rehberg & Gräf, 2002) with Bam HI and Hind III. pKK8 was generated utilizing a Kpn I/Bam HI fragment encoding amino acids 1- 813 of DdCP224 from pTOG38Bsr6 (Gräf *et al.*, 2000). In case of pKK9 a Kpn I/Bam HI fragment encoding amino acids 809-1392 (the C-terminal 584 aa) from pTOGCBsr3 (Gräf *et al.*, 2000) was used.
Additionally, an DdEB1-TAP vector with G418 resistance was created (pKK16) using a Hind/Xho fragment from pKK7 in the vector pA15GFPV18Sac, that consists of an N-terminal GFP under control of actin 15 promoter and a V18-promoter/G418 resistance cassette modified from pDiscGFPSSEB2 (Daunderer & Gräf, 2002).
For expression of a GFP-TACC-domain fusion (pKK14), a *Dictyostelium* cDNA library was used as a template for the amplification of a PCR product, which corresponded to base position 3611 to 4529 and was flanked with Sal I/Bam HI restriction sites. It was cloned into a modified pA6PGFP-SSEB vector (Rehberg *et al.*, 2005) with an additional GGSGG linker downstream from the GFP sequence.
To generate an MBP-TACC domain fusion (pKK20), the Bam HI/Sal I fragment containing the TACC domain from the GFP-TACC domain plasmid pKK17 was cut and ligated into the vector pMalC2 for the expression of MBP with TACC.
All other plasmids are briefly described in *Table 2* or in the respective section of the results.

No	Name	Description
	pGADT7	obtained from Clontech/BD Bioscience
	pGBKT7	obtained from Clontech/BD Bioscience
	pGEX-5x1	obtained from GE Healthcare
	pCL1	obtained from Clontech/BD Bioscience
	pGADT-T	obtained from Clontech/BD Bioscience
	pGBKT7-53	obtained from Clontech/BD Bioscience
	pGBKT7-Lam	obtained from Clontech/BD Bioscience
	pMalC2	obtained from New England Biolabs
	p1ABsr8	described in (Gräf *et al.*, 2000)
	pDiscGFPSSEB2	described in (Daunderer & Gräf, 2002)
	BindEB1	8.9 kb; pGBKT7 with linker ESSB+EB1 (1,6kb); Sac I/BamHI
	DdCPBait	9.6 kb; pGBKT7 with linker ESSB+2,3KB DdCP224C-term; Sac I/BamHI
	38Stop	9.7 kb; pGBKT7 with linker ESSB+2,4KB DdCP224N-term; Sac I/BamHI
pKK1	papg8GFPN-term	7698 bp; pA6PGFPV18+apg8 (369bp); Sal I/BamHI
pKK2	pE6GFPN-term	8076 bp, pV18A6PGFP-L-SSEB+EB1-6 (744bp); Sal I/BamHI
pKK3	pE26GFPN-term	8010 bp; pV18A6PGFP-L-SSEB+CalcineurinB (678bp); Sal I/BamHI
pKK4	pTAP1ABsr8	5799 bp; p1ABsr8+TAP (552bp); Nsi I/BamHI
pKK5	p38-27GFPN-term	7659 bp; pV18A6PGFP-L-SSEB+38-27 (327bp); Sal I/BamHI
pKK6	pE67GFPN-term	8022 bp; pV18A6PGFP-L-SSEB+EB1-67 (690bp); Sal I/BamHI
pKK7	pEB1TAP1ABsr8	7326 bp; pKK4+EB1 (1518 bp); Hind III/BamHI
pKK8	pDdCPNtermTAP1ABsr8	8238 bp; pKK4+N-terminal DdCP224 (2440 bp); Kpn I/BamHI
pKK9	pDdCPCtermTAP1ABsr8	9403 bp; pKK4+C-terminal DdCP224 (3622 bp); Hind III/BamHI
pKK10	papg8GST	5337 bp; pGEX4T3+apg8 (369bp); BamHI/Sal I
pKK11	pE6GST	5712 bp; pGEX4T3+E6 (744bp); BamHI/Sal I
pKK12	pE26GST	5636 bp; pGEX4T3+CalcineurinB (678bp); BamHI/Sal I
pKK13	p38-27GST	5285 bp; pGEX4T3+38-27 (327bp); BamHI/Sal I
pKK14	TACC domain NtermGFP	6567 bp; pIS76+TACC23 (830bp); Sal I/BamHI
pKK15	TACC domain NtermGFP	8148 bp; pV18A6PGFP-L-SSEB+ TACC23 (830bp); Sal I/BamHI
pKK16	EB1TAP G418	8298 bp; pA15pGFPV18Sac+EB1tap; Hind III/Xho I/Sal I
pKK19a	TACCKo	5150 bp; pIS102+TACC 5'utr (koKpn+koHind 800bp); Kpn I/Hind III
pKK19b	TACCKo	5150 bp; pIS102+TACC23 (TACC domain 800bp); Pst I/BamHI
pKK19	TACCKo	5950 bp; pKK19b+5'utr (koKpn+koHind 800bp); Kpn I/Hind III
pKK20	TACC domain NtermMBP	7412 bp; MAl C2+TACCdomain; Sac I/BamHI

Table 2 Plasmids generated for and used in this study

4 Biochemical and immunological methods

4.1 SDS-Polyacrylamide gel electrophoresis (PAGE)

Proteins were separated on discontinuous SDS-polyacrylamide gels (Laemmli, 1970). 12.5 % and 17.5 % polyacrylamide (PAA) gels were prepared. Gels were run in Biorad minigel System chambers at 15 mA per gel for 15 min and 30 mA per gel for 45 min subsequently. Probes and high molecular weight standard (Sigma) were mixed with 1/4 volume 5 x Laemmli sample buffer (Laemmli, 1970) or an equal volume of SDS sample buffer, incubated at 95°C for 5 min or mixed with a sample buffer containing urea and immediately loaded onto the gel.

Stock solution	**3 % stacking gel**	**12.5 % separation gel**	**17.5 % separation gel**
30 % Acrylamide	0.68 ml	3.70 ml	5.14 ml
1 % Bis-Acrylamide	0.50 ml	0.93 ml	0.64 ml
Separation buffer (2 M Tris/HCl, pH 8.7, 0.4 % SDS)	-	2.5 ml	2.5 ml
Stacking buffer (0.25 M Tris/HCl, pH 6.8, 0.4 % SDS)	1.0 ml	-	-
H_2O	1.78 ml	1.80 ml	0.72 ml
10 % APS	35 µl	45 µl	45 µl
TEMED	7 µl	9 µl	9 µl

Table 3 Components of SDS-Polyacrylamide gels with different concentrations of acrylamide

4.2 Coomassie and silver staining

4.2.1 Coomassie staining

Gels were stained for 30-60 min in Coomassie staining solution, rinsed with H_2O and destained with 10 % acetic acid. Gels were scanned (Epson 1200 Photo) and dried between two sheets of cellophane stretched by a plexiglas frame.

Coomassie staining solution

7.5 % acetic acid, 50 % methanol, 0.25 % Coomassie Brilliant Blue R250 (Sigma)

4.2.2 Colloidal Coomassie staining

This staining method is considerably more sensitive than the conventional Coonmassie R250 staining. Gels were fixed in 10 % TCA for at least 1 h and washed 3 x 10 min in H_2O. The Coomassie staining stock (2 g phosphoric acid (85 %), 10 g ammonium sulfate, 2 ml Coomassie G250 (5 %)) was mixed with ¼ volume methanol just before use and the gel was stained over night. Unbound color was removed by several washes in H_2O.

4.2.3 Silver staining

Silver staining methods are about 10-100 times more sensitive than various Coomassie Blue staining techniques. Consequently, they are the method of choice when very low amounts of protein have to be detected on electrophoresis gels.
Gels were fixed for 30 min up to over night in 40 % Ethanol, 10 % glacial acetic acid, 50 % deionized water, washed with deionized water for 5 min and sensitized for 30 min in a thiosulfate reagent containing 0.02 % sodium thiosulfate (in 37.5 % ethanol, 0.125 % Glutardialdehyde and 17 g Sodium acetate/250 ml). After a short washing in dH_2O, gels were incubated in a Silver nitrate reagent (0.2 % silver nitrate, 0.02 % formaldehyde (37 %)) for 20 min. After some short washing steps, developer (3 % sodium carbonate, 0.05 % formaldehyde (37 %)) was added until the desired staining strength was accomplished. The reaction was stopped by addition of 0.04 M EDTA and gels were preserved as described previously (4.2.1).

4.3 TCA precipitation of proteins

For precipitation of proteins to be subjected to Mass spectrometry in Würzburg, the sample was mixed with ¼ volume 50 % TCA (final concentration of 10 % TCA) and incubated on ice for 15 min. After centrifugation at 14,000 rpm for 10 min at 4°C, the pellet was washed with acetone and the washing steps repeated. The pellet was air-dried and either kept at –70°C until shipping or suspended in SDS sample buffer for immediate analysis in protein gels.

4.4 Western blots and immunostaining

Polyacrylamide gels were blotted with the semidry procedure using the buffer system of Kyhse-Anderson (Kyhse-Anderson, 1984), modified by the addition of 20 %

methanol to all three buffers. (Buffer 1 (300 mM Tris, 200 ml/l methanol), buffer 2 (30 mM Tris, 200 ml/l methanol), buffer 3 (30 mM Tris, 40 mM e-amino-n-capronic acid, 200 ml/l methanol).

Blotting was carried out for 1 h at 1 mA/cm^2 and blots were reversibly stained with 0.1 % Ponceau S (Sigma) in 5 % acetic acid prior to immunostaining. Marker bands were labeled on the blot before blocking in TBST (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05 % Tween-200) containing 3 % gelatine. Incubation with the primary antibody diluted in TBST/1 % gelatine was carried out for 1 h at room temperature, followed by washes in TBST (3 x 5 min) and incubation with the secondary antibody (1:10,000 dilution in TBST/1 % gelatine of anti-rabbit or anti-mouse antibodies coupled to alkaline phosphatase) for 40 min at room temperature. Blots were washed 3 x 5 min with TBST and once with TBS and color detection was carried out by 5-30 min incubation in AP reaction buffer (100 mM Tris/HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) supplemented with 4.5 µl/ml NBT (75 mg/ml stock in 70 % dimethylformamide) and 3.5 µl/ml BCIP (50 mg/ml in demethylformamide).

Alternatively, detection was performed using ECL detection system (AppliChem, Darmstadt) according to the instructions of the manufacturer. For this method, gelatine was substituted by skim milk powder and secondary antibodies coupled to peroxidase were used. Detection of luminescence was performed by incubation with x-ray films and subsequent development of these.

4.5 Determination of protein concentration

For rough estimations of protein concentrations the OD_{280} of a solution was measured, assuming about 0.7 mg/protein per 1 OD_{280} (a value which is most accurate for immunoglobulins).

For accurate measurements the Amido-black assay was carried out. 300 µl of staining solution (5.2 mg Amidoblack in 20 ml wash solution) were added to the protein, incubated for 5 min at room temperature, spun down and washed twice with wash solution and finally resuspended in 700 ml 0.1 M NaOH. The samples were measured at 615 nm and a BSA reference curve was used to determine the protein concentrations.

Wash solution

acetic acid/methanol 1:10

4.6 Purification of bacterially expressed, MBP-tagged proteins

0.5 l of the respective *E. coli* culture were grown to an OD_{280} of 1.0 in LB-Amp containing 10 mM glucose at 37°C. After induction with 0.3 mM IPTG cells were grown for another 2-5 h at 23-37°C, depending on the construct. Cells were harvested (10 min, 5,000 rpm, Sorvall rotor GSA, 4°C), resuspended in a small volume of TE buffer and frozen at –20°C. After thawing the cell suspension was sonicated and centrifuged for 20 min with 20,000 rpm (Sorvall SS34 rotor). The supernatant was filtered through a 0.8 µm polycarbonate filter and adjusted to 100 mM NaCl. The solution was then applied slowly (approx. 5 sec per droplet) to an affinity column containing 1 ml amylose resin. The resin was washed extensively with column buffer (200 mM NaCl, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA) or NHS coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and the MBP-tagged protein was eluted with the buffer of choice (either column buffer or NHS-coupling buffer) containing 10 mM maltose.
Purity of the preparation was assessed by SDS-PAGE.

4.7 Preparation of whole cell extracts, nuclei and centrosomes from *D. discoideum*

Whole cell extracts for SDS-PAGE were obtained by harvesting and washing cells as described above, resuspending the cell pellet in an equal volume of 0.5 x urea sample solution/separating buffer and boiling it for 3 min.
Nuclei were isolated from 1.5 l of a *Dictyostelium* culture with a density of 3-4 x 10^6 cells/ml. Cells were harvested and washed three times with Soerensen buffer, including 2 µM cytochalasin A in the last washing step. The cell pellet was resuspendend in 30 ml lysis buffer (10 mM Na-PIPES, pH 6.9, 2 mM $MgCl_2$, 10 % (w/v) sucrose, 0.25 % Triton X-100, 1 x protease inhibitor cocktail, 2 µM cytochalasin A) and cells were lysed by passage through a 5 µm mesh polycarbonate filter. Nuclei were pelleted by centrifuging at 2,500 g for 10 min at 4°C.
Centrosomes were isolated from isolated nuclei essentially according to Gräf et al. (1998). For this, the nuclear pellet was resuspended in 30 ml pyrophosphate buffer (100 mM Na-PIPES, pH 6.9, 2 mM $MgCl_2$, 30 % (w/v) sucrose, 40 mM tetrasodium diphosphate, 1 mM DTT, 1 % Triton X-100, 1 mM Na-ATP, 1 x protease inhibitor cocktail), vortexed for 1 min and centrifuged at 2,500 g for 10 min at 4°C. The

supernatant was supplemented with 0.6 ml of a heparin solution (10 mg/ml) and was incubated on ice for 5 min. The solution was then filtered twice through a 5 µm polycarbonate filter and was loaded on a first sucrose step gradient (two Beckman SW40 tubes containing 0.5 ml of 80 % and 1.5 ml of 50 % sucrose in gradient buffer: 10 mM Na-PIPES, pH 6.9, 2 mM $MgCl_2$, 0.1 % Triton X-100, 0.1 % 2-mercaptoethanol, 1 mM Na-ATP, 1 x protease inhibitor cocktail). After centrifuging at 55,000 g for 1 h at 4°C the centrosomes were recovered from the border between the two sucrose fractions (1 ml per gradient). The collected fraction was then loaded on a second sucrose density gradient (two Beckman SW50.1 tubes containing 0.5 ml of 80 %, 0.5 ml of 70 %, 1 ml of 55 % and 1 ml of 50 % sucrose solution). After centrifuging at 40,000 g for 1 h at 4°C two centrosome-containing fractions (0.6 ml and 0.8 ml) were collected from the bottom, with the second fraction containing less contaminating vesicles and DNA.

For immunofluorescence microscopy 2 µl of centrosomes were diluted in 0.5 ml PBS and were sedimented onto round untreated coverslips by centrifugation at 2,500 g for 20 min at 4°C. Centrosomes were fixed and stained as described in section 2.4.3. For SDS-PAGE nuclear and centrosome fractions were mixed with 1/4 volume of 5 x Laemmli buffer and boiled for 3 min.

4.8 Antigen preparation and immunizations

The fusion protein of DdTACC with MBP purified on amylose columns was of sufficient purity to be used directly for the immunization of two rabbits each (using about 1 mg of protein per rabbit). All immunizations were carried out by *Pineda Antikörper Service* (Berlin, Germany), using a standard protocol of five immunizations over 60 days.

4.9 Covalent coupling of antibodies and purified proteins to NHS-activated sepharose

Purified MBP-fusion proteins intended for coupling were washed and eluted directly in coupling buffer supplemented with 100 mM NaCl and concentrated to a final volume of 1 ml in YM30 Centricon devices or dialysed with coupling buffer overnight.

For coupling, 0.5 ml of NHS-activated sepharose (Pharmacia) were washed with ice cold 1 mM HCl and were immediately mixed with 1 ml of the protein solution

containing 0.5-5 mg of protein. The slurry was rotated in an Eppendorf cup for 2-4 h at room temperature or at 4°C over night. The resin was then washed several times alternating between buffers A (0.5 Methanolamine, 0.5 M NaCl, pH 8.3) and B (0.1 M acetic acid, 0.5 M NaCl, pH 4.0), allowing a 20 min incubation time in buffer A at half time, for complete saturation of all unoccupied binding sites. The resin was stored in 50 mM phosphate buffer, pH 7.0 containing 0.02 % NaN_3.

4.10 Affinity purification of antisera

Antisera were mixed with an equal volume of PBS, filtered through a 0.8 µm polycarbonate filter to remove particles and applied to sepharose columns containing the covalently coupled antigen (see section 2.3.11). The columns were washed extensively with PBS and specific antibodies were eluted with 100 mM glycine, pH 2.7 and were neutralized immediately by the addition of droplets of 1 M Tris/HCl, pH 8.7. For storage affinity purified antibodies were supplemented with 0.5 % BSA and 0.02 % NaN_3.

4.11 Tandem affinity purification of *D. discoideum* protein complexes

The following protocol was modified from the one described by Rigaut (Rigaut *et al.*, 1999). To prepare a sample for subsequent mass spectrometrical analysis, approximately 5 x 10^8 cells (200 ml cell culture) were used to prepare a cytosolic protein extract. Cells were centrifuged at 1,400 rpm for 5 minutes and washed with phosphate buffer two times. The pellet was resuspended in 8-10 volumes (10-12 ml) of IPP150 buffer (10 mM Tris-Cl, 150 mM NaCl, 0.1 % NP40, pH 8.0) containing protease inhibitors (1 mM Pefabloc SC, 25 µg/ml leupeptin, 10 µg/ml tosyl-arginine-methylester, 10 µg/ml soybean trypsin inhibitor, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM benzamidine, all from Biomol, Hamburg, or Sigma, Deisenhofen, Germany) (Gräf, 2001a), cytochalasin A (final concentration 2 µM) and ATP (final concentration 1 mM). The suspension was passed three times through a 5 µm Nucleopore filter (Whatman) to lyse the cells. Cell debris was removed by centrifugation at 14,000 x g at 4°C for 15 minutes and 10 ml of the supernatant protein extract (containing about 4-6 mg/ml of protein) were added to 250 µl of IgG agarose beads (Amersham, Uppsala, Sweden), washed beforehand with 15 ml of IPP150 buffer, and incubated on a rotator for 2.5 h at 4°C in a 15 ml centrifuge tube to allow binding of the protein

A part of the TAP-tag to the beads. The suspension was then added onto a Poly-Prep® chromatography column (BIO-RAD, Hercules, CA, USA) and washed with 30 ml IPP150 followed by 10 ml of TEV cleavage buffer (10 mM Tris-Cl, 150 mM NaCl, 0.1 % NP40, 0.5 mM EDTA, 1 mM DTT (added immediately before use), pH 8.0). Next, 100 units (10 µl) of AcTEV protease (Invitrogen, Karlsruhe, Germany) were added in 1 ml TEV cleavage buffer, and the protein was eluted from the beads during 2 h of rotation at 16°C.

The eluate (1 ml) was recovered under addition of 200 µl TEV cleavage buffer, and supplied with 3 ml calmodulin binding buffer (10 mM Tris-Cl, 150 mM NaCl, 0.1 % NP40, 10 mM β-mercaptoethanol, 1 mM Mg-Acetate, 1 mM imidazole, 2 mM $CaCl_2$, pH 8.0) and 3 µl 1 M $CaCl_2$.

The mix was added to the second affinity column with 250 µl of a calmodulin bead suspension (Stratagene, Amsterdam, Netherlands), washed beforehand with 7.5 ml of IPP150 calmodulin binding buffer, and rotated for 1 h at 4°C. After removal of the solution, the beads were washed with 30 ml of IPP 150 calmodulin binding buffer and eluted with 1 ml of IPP150 calmodulin elution buffer containing EGTA (10 mM Tris-Cl, 150 mM NaCl, 0.1 % NP40, 10 mM β-Mercaptoethanol, 1 mM Mg-Acetate, 1 mM imidazole, 2 mM EGTA, pH 8.0).

For SDS gel electrophoresis the eluted proteins were precipitated with trichloracetic acid (10 % final concentration) and subsequently washed twice with acetone. The dried pellet was kept at –70°C until further analysis.

4.11.1 Protein electrophoresis and mass spectrometry

Protein electrophoresis and mass spectrometry was performed by Y. Reinders in the Lab of Albert Sickmann at the R. Virchow Institute for Biomedicine in Würzburg and is added to this thesis for a complete description of the generation of the data used in this thesis. One dimensional polyacrylamide gel electrophoresis: Proteins were solubilized in LDS-sample buffer (Invitrogen, Karlsruhe, Germany) and reduced with DTT at 90°C for 10 minutes. Samples were separated on 4-12 % precast BIS-Tris gels (NuPAGE™ Invitrogen, Karlsruhe, Germany). Gels were silver stained according to the protocol of Blum et al. (Blum et al., 1987).

In-gel digestion and peptide extraction: Sample preparation was performed according to the protocol of Shevchenko et al. (Shevchenko *et al.*, 1996). Briefly samples were washed twice using 50 mM ammonium hydrogen carbonate and 25 mM ammonium

hydrogen carbonate with 50 % acetonitrile, respectively. Proteins were reduced using 10 mM DTT for 30 minutes at 56°C and subsequently alkylated by incubation with 20 mM iodoacetamide at room temperature for 30 minutes. Furthermore, samples were washed as described before. Gel bands were dehydrated in a vacuum centrifuge and rehydrated with 12.5 ng/µl trypsin in 50 mM ammonium hydrogen carbonate. Digestion was performed at 37°C over night. The resulting peptides were extracted by application of 15 µl of 5 % formic acid for 10 minutes.

LC-MS-Analysis and data interpretation: For reversed phase separation 0.1 % formic acid as solvent A and 0.1 % formic acid in 84 % acetonitrile as solvent B were used. Separation was performed using a nano-HPLC-system (Dionex, Idstein, Germany). After gel electrophoresis, samples were pre-concentrated onto a 300 µm I.D. C_{18}-PepMap™ column of 1 mm length at a flow rate of 25 µl/min. Separation was performed using a 75 µm I.D. column (C_{18}-PepMap™, 15 cm length, 3 µm particle size and 100 Å pore size) with a flow rate of 250 nl/min. Therefore, a binary gradient starting at 5 % solvent B rising to 50 % in 30 minutes was used. After elution the column was rinsed with 95 % solvent B for 10 minutes and subsequently equilibrated with 5 % solvent B for 20 minutes. Peptides were directly eluted into an ESI-mass spectrometer (LCQ^{TM} Deca XP^{plus}, Thermo Electron, Dreieich, Germany). MS-acquisition duty cycle was set up to 1 s survey scan followed by 3 dependent scans, each approximately 1 s. Mass spectra were transformed into peak lists in mgf-format using a in-house software solution raw2dta. Default values for generating mgf-files were applied and data was processed using the search algorithm MASCOT™ (version 1.9, Matrixscience, London, UK). Database searches were accomplished using the DictyBase database (version October 2004, http://www.dictybase.org). As fixed modification carbamidomethylation of cysteines was selected and oxidation of methionine as optional modification. For MASCOT™ the minimum score was set to 35 for each peptide. All spectra were verified manually in order to avoid false positive hits derived from the search algorithm.

4.12 Immunoprecipitation

Immunoprecipitation was performed essentially as described previously (Hestermann & Gräf, 2004). In brief, cells of a density of about 5 x 10^6 cells/ml were washed in phosphate buffer and lysed in 10 x the amount of lysis buffer (50 mM Hepes, 100 mM NaCl, 4 mM EGTA, 2 mM $MgCl_2$, 10 % sucrose, 0.3 % NP40, 1 x protease inhibitor

cocktail (Gräf, 2001a). A cytosolic extract was obtained after centrifugation at 14,000 x g for 15 min at 4°C. After incubation of 0.6 ml of cytosolic extract with 10 µg of purified antibodies or 2 µl of antiserum for 1 h at 4°C, 20 µl of Protein G beads (50 % slurry pre-incubated with 0.1 % BSA in Tris-buffered saline) were added for a further incubation for 1 h at 4°C in a rotator. Beads were washed 4 times with lysis buffer, resuspended with 30 µl of SDS sample buffer (10 % SDS, 125 mM Tris/HCl, pH = 6.8, 50 mM DTT, 5 % glycerol) and subjected to SDS gel electrophoresis and Western blotting.

4.13 GST-Pulldown

For GST-pulldown experiments, *E. coli* strains expressing the GST tagged proteins were grown overnight, diluted to 100 ml and grown to an OD_{600} of 0.8. Then 840 mM IPTG was added for induction and cultures were incubated for another 5 h at 21°C. The pellets were resuspended in 2 ml phosphate buffer and frozen at –20°C.
After thawing, cells were lysed with short ultrasound pulses and the solution supplemented with 70 µl 10 x PBS and 1 mM DTT. After centrifugation at 14,000 rpm for 30 min, the supernatant was added to 40 µl of 30 % glutathione sepharose (GE healthcare) and rotated for 30 min at 4°C. Beads were washed 3 x with PBS plus 1 mM DTT plus 2 mM $MgCl_2$. In parallel, *D. discoideum* cells expressing GFP-fusions of the proteins of interest were lysed in Fish buffer supplemented with 1 x protease inhibitor cocktail (Gräf, 2001a), cytochalasin A (final concentration 2 µM) and benzamidine (final concentration 1 mM). Cells were pelleted at 14,000 rpm for 10 min and 500 µl of supernatant was incubated for 1 h at 4°C with the beads prepared as described above. Finally, beads were washed with Fish buffer plus 1 mM DTT for 4 times and resuspended with 30 µl of SDS sample buffer (10 % SDS, 125 mM Tris/HCl, pH = 6.8, 50 mM DTT, 5 % glycerol) and subjected to SDS gel electrophoresis and Western blotting.

5 x Fish buffer

25 % glycerol, 200 mM Tris/HCl pH 7.4, 250 mM NaCl, 5 % NP40, 10 mM $MgCl_2$

4.14 Isolation of *Dictyostelium* centrosomes

To obtain centrosomes of sufficient purity for immunoflourescence, an improved protocol according to Gräf et al. (Gräf *et al.*, 1998) was used. A cell suspension in AX-medium (ca. 5 x 10^9 cells with a density of ca. 2 - 4 x 10^6 cells per ml) was centrifuged for 5 min at 500 x g at 4°C. All steps were carried out on ice or in cooled centrifuges of 4°C. The resulting pellet was washed 3 x in phosphate buffer (ca. 5-fold volume of the pellet) and spun down for 5 min at 500 x g at 4°C. 2 µM cytochalasin A were added to the phosphate buffer prior to the last washing step. Cells were resuspended in lysis buffer and vortexed for 1 min. The suspension was filtered through a 5 µm Nucleopore-Filter (Nucleopore, Costar GmbH, Bodenheim) and immediately centrifuged for 10 min at 2,500 x g at 4°C. The resulting pellet was resuspended in pyrophosphate buffer, vortexed for 1 min and immediately centrifuged for 10 min at 2,500 x g at 4°C. Heparin solution was added to the supernatant and placed on ice for 5 min. The mixture was carefully loaded onto a succrose gradient containing 0.5 ml of supplemented 80 % sucrose solution and a layer of 1 ml of supplemented 50 % sucrose solution in Beckman SW40-tubes and centrifuged for 1 h at 40,000 x g (21,000 rpm) at 4°C. After centrifugation, all liquid up to the upper half of the 50 % fraction including the faint white band which is often visible was removed and all remaining liquid including the small pellet was resuspended and passed twice through a 27 gauge needle using a 1 ml syringe and supplemented with the 0.5-fold volume of supplemented gradient buffer. This suspension was then loaded onto the 2nd gradient containing 0.5 ml 80 % sucrose, 1 ml 55 % sucrose and 1 ml 50 % sucrose in Beckman SW50.1 gradient tubes and centrifuged for 1 h at 40,000 x g (21,000 rpm) at 4°C. Fractions were collected from the bottom using a peristaltic pump and a glass capillary (ca. 200 µl per min). The glass capillary should not touch the pellet that might have formed. The volume of the first fraction which is the centrosome fraction was ~700 µl. If there was a clear pellet it usually contained intact centrosomes as well, but these were heavily contaminated with DNA. For the preparation of GFP-tagged centrosomes, β-mercaptoethanol or its substitute DTT were left out, since they destroy GFP fluorescence. Centrosomes were either immediately used for SDS page or centrifuged onto coverslips for immunofluorescence or immediate analysis by microscopy or frozen at –70°C in gradient buffer.

Lysis buffer

100 mM Na-PIPES, pH 6.9, 2 mM $MgCl_2$, 10 % sucrose;
add prior to use: 0.25 % Triton X-100, 1 x protease inhibitor cocktail, 2 µM cytochalasin A

Pyrophosphate buffer

100 mM Na-PIPES, 2 mM $MgCl_2$, 30 % sucrose; add prior to use: 40 mM tetra-sodium diphosphate/HCl, pH 6.9 (M_r = 446 g/mol), 1 mM DTT, 1 % Triton X-100, 1 x protease inhibitor cocktail

Protease inhibitor cocktail

1 mM Pefabloc SC, 25 µg/ml leupeptin, 10 µg/ml tosyl-arginin-methylester, 10 µg/ml soybean trypsin inhibitor, 1 µg/ml aprotinine, 1 µg/ml pepstatine, 2 mM benzamidine, 1 mM Na-ATP

Gradient buffer

10 mM Na-PIPES, 2 mM $MgCl_2$; add prior to use: 0.1 % Triton X-100, 0.1 % β-mercaptoethanol or DTT

Sucrose solutions

80 % sucrose in 10 mM Na-PIPES/2 mM $MgCl_2$; add prior to use: 0.1 % Triton X-100, 0.1 % β-mercaptoethanol, 1 x protease inhibitor cocktail.

5 Cell biological methods

5.1 Indirect immunofluorescence microscopy on whole *D. discoideum* cells and isolated centrosomes

Cells of a logarithmically growing *D. discoideum* culture were diluted to 1 x 10^6 cells/ml with HL5 medium and allowed to settle on a round coverslip. After about 20 min the medium was removed and cells were fixed with the respective fixation method.
Isolated centrosomes were centrifuged onto round coverslips (see section 2.3.10) and fixed like whole cells.

For this work the following fixation procedures were used:

Formaldehyde fixation

3.7 % (v/v) formaldehyde, 0.5 % Triton-X-100 in 50 % PHEMbuffer, 5 min fixation time.

Methanol fixation

100 % methanol (-20°C), 5-10 min fixation time at –20°C.

Glutharaldehyde fixation

20 % or 50 % PHEM-buffer (100 % = 60 mM PIPES, 25 mM HEPES, 8 mM EGTA, 2 mM $MgCl_2$, pH = 6.9), 0.5 % Triton X 100, 0.5 % Glutaraldehyde (25 % stock solution); 5 min fixation time at RT. Cells were washed 2 x 5 min with 17 mM phosphate buffer and 1 x 10 min with 17 mM phosphate buffer and 1 mg/ml freshly added Borhydride.

Formaldehyde-Glutaraldehyde

3.7 % Formaldehyde, 0.05 % Glutaraldehyde, 0.5 % Triton X 100, 50 % PHEM-buffer. 5 min fixation time at RT. Cells were washed 2 x 5 min with 17 mM phosphate buffer and 1 x 10 min with 17 mM phosphate buffer and 1 mg/ml freshly added Borhydride.

Immunolabelling was carried out by incubating the fixed samples with one or a mixture of two different (for double staining) primary antibodies diluted in PBS, 1 % BSA, 0.1 % NaN_3 at room temperature for 1 h. Unbound antibodies were removed by 3 x 5 min washes with PBS and the samples were incubated for 1 h with the respective secondary antibodies (FITC, Cy3, Alexa 488 or Alexa 568 conjugates, diluted as recommended by the manufacturer) avoiding light exposure. The coverslips were finally washed three times, including DAPI (1 µg/ml) in the second washing step to visualize nuclear and mitochondrial DNA. For visualization of DNA under the confocal microscope, TO-PRO3 (Molecular Probes, excited at 633 nm) was added to the second antibody (dilution 1: 1,000). Coverslips were embedded on a microscope slide with 5 µl anti-bleach (10 % PBS, 1 % phenylenediamine, 89 % glycerol) and the edges were sealed with nail polish. Alternatively, coverslips were embedded in Pro Long (Molecular Probes) and left to polymerize. Standard immunofluorescence microscopy preparations were viewed through a Zeiss Axiovert 200M microscope equipped with a 100 x/1.3 objective and DAPI, rhodamine and fluorescein filter sets. Images were recorded with a “AxioCam Mrm“ camera connected to a computer running the program Axiovision 4.3

5.2 Confocal microscopy

For confocal analysis preparations were viewed on an inverted microscope (Zeiss Axiovert 200M/510Meta Laser Scanning System) equipped with a 63 x/1.4 objective.
For the imaging of live cells, laser intensity was reduced and cells were viewed under an agar-overlay (Fukui, 1987).
Images were acquired using the Zeiss 510Meta confocal imaging system, in part processed with Huygens 2.2 Deconvolution-Software (Bitplane AG, Zürich, Switzerland) using a calculated theoretical „Point Spread Function“ and a „Maximum Likelihood Estimation Algorithm” and later on transferred to a Macintosh computer and analyzed using NIH image and Adobe Photoshop 7.0.

III Results

The aim of this work was to find new interactions of DdCP224 and DdEB1 and establish an appropriate method to do so.
To obtain more detailed knowledge about the protein complexes and interactions present both at the centrosome as well as at the microtubule plus ends screenings for interactors of the known and well-characterized proteins DdCP224 and DdEB1 (Gräf *et al.*, 2000; Gräf *et al.*, 2003; Rehberg & Gräf, 2002) were carried out.
Since a *Dictyostelium discoideum* cDNA library in yeast was readily available (courtesy of Jan Faix, unpublished), yeast two-hybrid screening was the first method utilized to look for interactors.

1 Screening for interactors of DdEB1 and DdCP224 employing the yeast two hybrid system

The fact that in many eukaryotic organisms transcription factors are modular, led to the development of a system to test for protein-protein interaction. For example, in *S. cerevisiae*, the GAL 4 promotor can be separated into its DNA-binding and DNA-activation domain that form a functional transcription factor upon sufficient proximity. To enable the activation of reporter genes used to monitor the interactions the protein of interest (bait) is fused with a GAL4 DNA-binding domain, whereas the cDNA library plasmids express the GAL4 activation-domain. (Fields & Song, 1989) In the yeast two hybrid system used in this study (Clontech's Matchmaker) interactions lead to the activation of a set of reporter genes, ADE2, HIS3, lacZ and MEL1, which can be monitored by growth on the respective selection media and a β Galactosidase assay. Additionally, all clones containing both the library and bait plasmids should be auxotroph for leucine and trypsin due to the presence of cassettes for their expression on the plasmids (leucine on the library plasmid and trypsin on the bait plasmid). In search for interactors of DdEB1 and DdCP224, a *Dictyostelium discoideum* library in the pGADT7 vector *(Figure 4*) was utilized. For its generation, a combination of oligo (dT) and random priming were used. The advantage of an oligo (dT)-primed, directionally cloned library is that one-third of all the clones will be

expressed in the correct frame, thereby maximizing the chances of finding a positive interaction. The disadvantage of a directionally cloned library is that the 5' ends of very long transcripts and hence, the amino terminus of some library proteins may be underrepresented. On the other hand, all portions of the transcripts, including the 5' end, are equally represented in a randomly primed, not directionally cloned library; however, only one-sixth of the clones will be expressed in the correct frame. As baits, a full-length DdEB1 construct in the vector pGBKT7 was used. DdCP224 was split into two parts, since the C-terminal 460 amino acids of DdCP224 are sufficient for centrosomal binding (Hestermann *et al.*, 2002), whereas a construct consisting of the N-terminal 813 amino acids localizes to the cell cortex (Hestermann & Gräf, 2004). All constructs used as baits are shown in *Figure 4*.

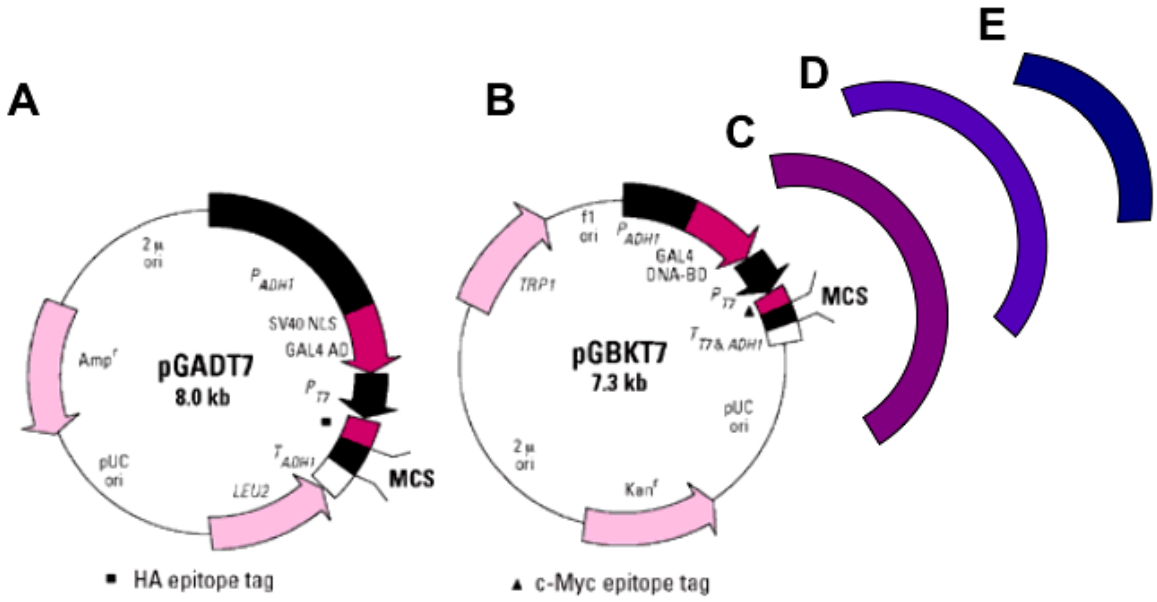

Figure 4 Maps of pGADT7, pGBKT7 and bait constructs. A pGADT7 expresses the cDNA library fused to the GAL4 activation domain. **B** pGBKT7 expresses bait proteins fused to the GAL4 DNA-binding domain. MCS of pGBKT7: Nde I, Nco I, Sfi I, EcoR I, Sma I, Xma I, BamH I, Sal I, Pst I. (modified from: http://www.clontech.com/clontech/archive/JUL99UPD/MMVectors.shtml) **C** For the N-terminal part of DdCP224, the N-terminal 2.4 kb were integrated into the vector. **D** The C-terminal 2.3 kb of DdCP224 were used as second bait. **E** For DdEB1, a full-length construct was used. All baits were integrated using the Sac I/BamH I cloning sites of the modified vector pGBKT7 with an EcoR I, Sac I, Sma I, BamH I linker.

1.1 Test for autoactivation of baits

In order to exclude a reaction of the bait proteins by themselves due to an intrinsic DNA-binding or transcriptional activation activity, a test for autoactivation in yeast was conducted prior to the actual yeast two-hybrid screening.

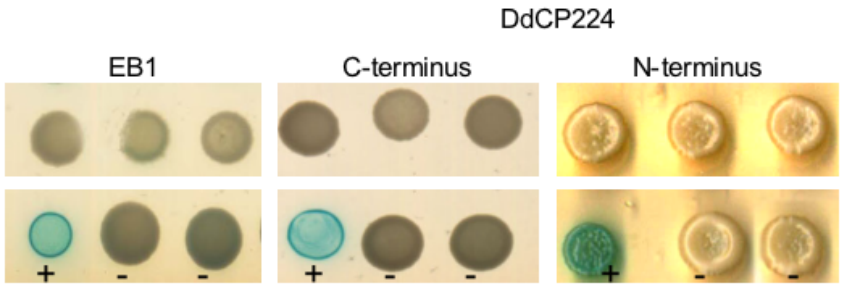

Figure 5 Test of baits for autoactivation. All bait plasmids were tested for autoactivation. Bait vectors were transformed into yeast strain Y187. Upper rows show growth of three independent yeast clones for each of the bait plasmids. Lower rows show controls provided by the manufacturer. A positive result of the β Galactosidase essay results in a blue colouring of the colony as can be seen with the positive control.

Figure 2 shows that all baits displayed no autoactivation and are thus suitable for a screen for interactors.

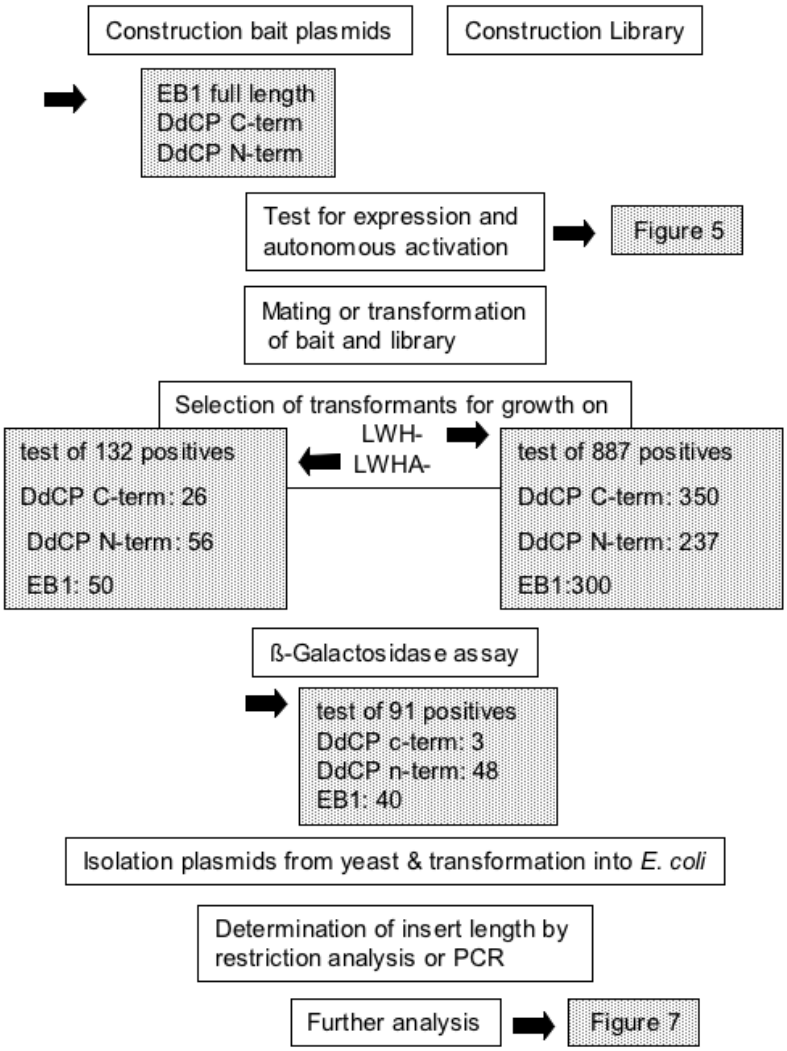

Figure 6 Flow chart of yeast two hybrid screen. Steps of a yeast two hybrid screen are shown in white boxes; specific information on the screen carried out in this study are shaded in grey and connected to the appropriate steps by arrows.

The strain containing the library was transformed separately with all three bait constructs by high efficiency transformation (see materials and methods). Clones were first grown on LW^- medium where all clones containing a set of bait and library plasmids should be able to grow and then selected on LWH^- (medium stringency) and $LWHA^-$ (high stringency) media to select for activation of the reporter genes ADE2 and HIS3, as shown in *Figure 6*.

1.2 Selection of clones of interest

To distinguish between false positives and putative interactors, the numerous clones growing on medium stringency LWH^- medium (887 in total, 350 resulting form the DdCP c-term bait, 237 resulting from the DdCP n-term bait and 300 detected from the DdEB1 bait) only 132 (26 from DdCP c-term, 56 from DdCP n-term and 50 from DdEB1) were still able to grow under more stringent conditions on $LWHA^-$ media and were subjected to a β Galactosidase assay.

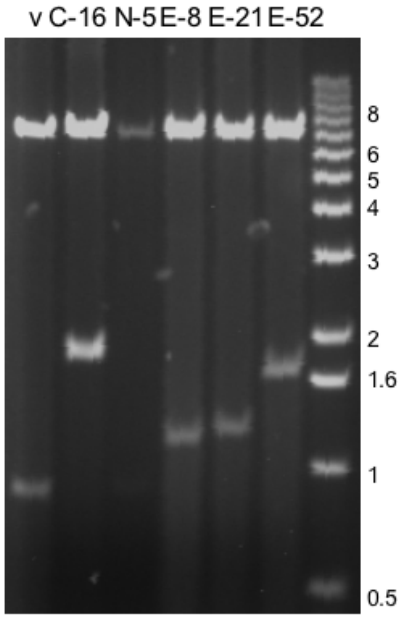

Figure 7 Analysis of insert length by restriction analysis. Gel electrophoresis of library plasmids subjected to enzyme restriction with Hind III. Lane 1 shows the restriction pattern of the empty vector pGADT7, lanes 2-6 show the patterns of various selected clones. Sizes of marker bands are given in kb. Size shift compared to the insert of the empty vector indicates the length of the cDNA insert in the library plasmids of the selected clones.

Performing a β Galactosidase assay, only 91 (3 from DdCP c-term, 48 from DdCP n-term and 40 from DdEB1) positives could be identified. Those were isolated from yeast and transformed into *E. coli*. Insert length of the library plasmids was determined by restriction analysis as shown in *Figure 7* or by PCR.

1.3 Sequence analysis of putative interactors

Putative interactors were sequenced and sequences were subjected to blast search both at DictyBase (http://www.dictybase.org) to identify the surrounding sequences as well as at http://www.ebi.ac.uk/blastall/index.html for a general search for homologies. From the clones sequenced and analyzed, most were very short and contained poly-A- stretches. 19 other clones were probable false positives, 7 of which were homologue to/or ribosomal sequences, the other 12 had stop codons in all reading frames and also at the beginning of the sequence or were otherwise classified as false positives since they displayed no hints for their being part of an open reading frame.

5 positive clones sequenced promised to be putative interactors since they contained open reading frames and were therefore subjected to further analysis.

A

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1/1                                 31/11                               61/21                               91/31
AAA AAT ATG GGT TTT ATT ATT ACG CAA CAC ATT CAA AAA TAA AAA ATT AAA ATA AAA ATA ATT ATG TTC AAA ATT TTA CCG CCT GCA AAG AAA AAA AAC AAA TCA TAA CAA ATT TGT TTA
121/41                              151/51                              181/61                              211/71
TTT TTT TTT TTT TTT TTT ATT TTA TTT TTC TTT TTT TTA TTT TTC TTT TTT TAT TTT TCT TTT TTT TTC AAA AAA CGG TTT AAC AAA AAT ACA CAA TTT AAA TGT GTT GTA TTG CGT AAT
241/81                              271/91                              301/101                             331/111
AAA AAA AAA AAA ATC AAA AAA AAA AAT ATC TTT TGT GAA GTT TGC ACA TCG ACG AAC TAT TTT CCT TCC CTT TTC GGT TTT TTT TTT AAA TTT ATT ATT TTG ATT ATT TTA AAT TTT TTT
361/121                             391/131                             421/141                             451/151
TTT TTA AAA TTA TTT TTT TGA TTA TTT TCC TAT TCT TTT TTT TTT TTT TTT TTT TTA GAT TTA TAA TTT TTA TTT TAT TTT CTT CCT TTT TTT TTT TTT CTT TTT TTT TTT TTT TTT
481/161                             511/171                             541/181                             571/191
TTT TTT TTT TTT TTA CAA TTT AGT TTC TTT GAA GGT TCA TTG AAA TAT ATA CAA TAT GAA AAA ATT CGA GTC TAT ATT TAA CCA CAT TAT CTA GAT AAA TAG TTC GCG AGC AAA ACA CGA
601/201                             631/211                             661/221                             691/231
AAG TAA CTT TTT TTA TTT TTA AAG AAA TGA ACA ACC TAC TTT ACT AAG AAA TTG ATC AAA TGG TTT TTT TAA TTT ATT TTT ATT TTT TTT TAT TTT TTT TTT TTT TTT TTT AAA AAA ATT
721/241                             751/251                             781/261                             811/271
TTC TCA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TTT ATG AGA CAA TTT TTT AAT ATT TTA ACC AAA TTT TTT TTT TTT TTT ATT TTA AAA ATA AAT ATA
841/281                             871/291                             901/301                             931/311
CTC TTT ACA CTC TTT GCT TTT TAA TTA TCT TAA TTG TAA GTT TCC ATC ATT TAT TTA TTT TTT GTT TTA TTA ATT TTC TTT TTT TTT TTT TTT TTT TTC ATA CAA TAA TAA ATT TCA
961/321                             991/331                             1021/341                            1051/351
TAA ATT AAT AAT TTT TAA TTT AAA GAA CCA AAT ACA GAA ATG ACA ATA ACC AAT TAT CCA TTT GTT ACT GGT TCA AAC CTT ACA TCA CCA TGC GAT ATT CCT CGT GTC GCT CTT CTT GCT
                                                    M   T   I   T   N   Y   P   F   V   T   G   S   N   L   T   S   P   C   D   I   P   R   V   A   L   L   A
1081/361                            1111/371                            1141/381                            1171/391
TAT ACA AAC CAA AGT CTT GCA TTA AAT GCT GCT GGA GGT ACA ATT GAA AGC GCA TCA GAT TTA TTC TCA ATC ACT CTT TGC AAA TAT TAT TTG AAT AGC GTC GTT TCT CTC TCT CCA ACC
Y   T   N   Q   S   L   A   L   N   A   A   G   G   T   I   E   S   A   S   D   L   F   S   I   T   L   C   K   Y   Y   L   N   S   V   V   S   L   S   P   T
1201/401                            1231/411                            1261/421                            1291/431
AAT GTA TTT GGT GGT GTT GCA CAT TAT TCC AGT TTA ACA ACA CTT ATG AAT AAT CTT GAC TCT GCC GCT GAT ACC ATT CTC AAT GCA CTC TAC GCA TCC ATC AAT ACT TGT GGT ACT TTC
N   V   F   G   G   V   A   H   Y   S   S   L   T   T   L   M   N   N   L   D   S   A   A   D   T   I   L   N   A   L   Y   A   S   I   N   T   C   G   T   F
1321/441                            1351/451                            1381/461                            1411/471
ACT GAT TTG ACT GCA ACA AAA TTC GAC ACA CTC TCA ACA GCC TTT AGT GGT TAT AGA ACA ACT ATT TTA TCA CTC CAA ACT AGT TCT AAT ACT TTA AAA ACA GCA ATC ACT ACA ACC AGA
T   D   L   T   A   T   K   F   D   T   L   S   T   A   F   S   G   Y   R   T   T   I   L   S   L   Q   T   S   S   N   T   L   K   T   A   I   T   T   T   R
1441/481                            1471/491                            1501/501                            1531/511
GAC AGT TTA ACA TTG ACC ACT GAC ATT TAT AAC ACT GTA AAA ACA TGT TAC ACA AAT GTT ATC ACT GCT TTA GAA GGT TTG AGT GCT CGT TTG GGT CAA GTT GCA TCT TTA CTT AAT ACT
D   S   L   T   L   T   T   D   I   Y   N   T   V   K   T   C   Y   T   N   V   I   T   A   L   E   G   L   S   A   R   L   G   Q   V   A   S   L   L   N   T
1561/521                            1591/531                            1621/541                            1651/551
CAT AAC GCA AAT TTC CCA ACA TTT AAG GAT AAT GTT ACC TCT TAT ACT GAT CCA GAA GGT CCA GGT GAT CAA AGC AAT TAT ATG TCT TCA ATG AAC TAT TCC ATG GTT ACT TAT TTG ATT
H   N   A   N   F   P   T   F   K   D   N   V   T   S   Y   T   D   P   E   G   P   G   D   Q   S   N   Y   M   S   S   M   N   Y   S   M   V   T   Y   L   I
1681/561                            1711/571                            1741/581                            1771/591
AGC TCA AAT ACC ATT TTT TCA AAA CTT TAT TTT TAT AAA AGA TTA CGT GGT GTT ATC TTT TAA ATA TAT TAA ATT TAA TTT AAT TAA ATA GAT CTG TAA TTT TAG AAA AGA TTC ATA AAA
S   S   N   T   I   F   S   K   L   Y   F   Y   K   R   L   R   G   V   I   F   *
1801/601                            1831/611                            1861/621                            1891/631
ATA AAT AAA TAA AGA AAT TGT CTT CTT TTA AAT TTT TAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AGT TTT TAT AAT TTT ATT CAC AAT TAG CGC CAC CCT CAC CAA AAA ATC TTG AGA
1921/641                            1951/651                            1981/661                            2011/671
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2041/681                            2071/691                            2101/701                            2131/711
AAT TAA AAA AAA AAT TAA AAA AAA ATA AAA TTA AAA TAA AAA AAA AAA AAA AAA GCC AAC ATC ACT TTT TTT AAT TTT TTT TTT TAA TGT TTT TTT CGC TTG TTG TAA AAA AAA AAA AAA
2161/721                            2191/731                            2221/741                            2251/751
AAA AAA AAA AAA AAA ATG ATA AAT CAA CCA AAT TAT TTA TTA TTT TTT TTA TTA TTT ATT TAT TTA AAT ATT TTG GTT GTG AAT TCA GAT GAA AAT ATC TTT GTA AAT TAC AAT GTT AAT
2281/761                            2311/771                            2341/781                            2371/791
AGA AAT TAT ATT AAA CCA GAG GAA TGT GGT TCA TTG GAA ATT CCA TGT GTT TCT TTA GAA GAT GCT GGA AAT AAA GCA ATT TTA GAA AAT AAA AAT GGA ATT GAA AAA ATT ATT ATC AAT
2401/801                            2431/811                            2461/821                            2491/831
ATT ATT GGA AAT ATC AAT GGG TCT ACA TCG GCA TCA TTT GGT AAT TTA TAT AGT TTT TGT GGT GTT TTG GAA ATT AAT TCC TAT GAC ACT CAA AAA ATA ACG ATT GAT GGA TCA AAT TCA
2521/841                            2551/851                            2581/861                            2611/871
AAT AAC CCA TTT ATT AAT ATT CAA GAG AAT CAG TAT GAA AGT TGT TTA ATT CCA AAA AAA TTT CCG ATT AGA AAT ATT AAT TTT AAA AAC TGG GAG CAA ACA TTG GTA AAT ATT AAT ATC
2641/881                            2671/891                            2701/901                            2731/911
AAC CAA GAA ACA AAT CAA ACT GTA AAT GTG GAA TCA CTA TTT ATA TAT TTT ACC AAT AAT ACC ATG AAT TCT TTA AGT AAT ATT ATT TCT GTT TAT CCA AAG A
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B

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/1                                  31/11                                  61/21                                  91/31
GTC ATC GTT TAG GTG TAA ACT ATC AAC AAA TCC CAG TCA ATT GTC CAT TCG CCG TAA AGG GTG GTG TTA AAA ACT ACC AAC GTG ATG GTT TCA TGG CTG TCA ATG GTA ATG GTG GTA AAG
121/41                              151/51                                 181/61                                 211/71
GTC CAA ACT ATC AAC CAA ACT CTT TCG GTG GTC CAG AGC CAC ATC CAG AAT TTG CTC AAC ATA AAT TTG ACG TCT CTG GTT TCG CTG CTC GTC AAC CAT ACA ATC ATC CAA ATG ACG ACT
241/81                              271/91                                 301/101                                331/111
TTG TTC AAC CAG GTG ATT TAT ATA GAC TCA TGT CTG AAG ATG CCA AAT CTC GTT TCG TCT CTA ATT TAG TCG GTC ACA TGT CCG GTG TCA CCA TCA AAG AAA TTC AAG TTC GTG CCG TTT
361/121                             391/131                                421/141                                451/151
CAA ACT TTT ACA AGG CTG ACA AAG ATT TAG GTG CTC GTT TAT GTA AGG GTC TTG GTA TTG ATG TTA ATG ATG TCA TCA AAT TCG CTG CTA GAT CAA ACT TGT AAA TCA ATA TAT TAA AAA
481/161                             511/171                                541/181                                571/191
TAA TAA AAA AAT AAA AAA ATA AAA AAA AAT AAA AAA TTA AAT CTC AAT CTT TTT TTA ATT ATA AAA AAA AAT AAA AAA GAA AAA AAT TAA AAA AAA ATA AAA AAG AAA AAT TAA AAA AAA
601/201                             631/211                                661/221                                691/231
AAA ACA AAA AAC AAA AAA CAA AAA ACA AAA AAC AAA AAA CAA AAA AAA AAA AAA AAA AAA AAA TCA AAC AAA TGG ACC ATA GTA TGA AAG TTT GAT CAA ACT TGT TGA CAT ATT TTT GCA
721/241                             751/251                                781/261                                811/271
TCA CCT ATA GTG TAG TAT ATG AAA TGT TGT TGT ATG TAC AAA ATT TAA ATA AAA AAA AAA AAA AAT CAA ACA AAT GGA CGA TAG TAT GAA AGT TGA CAT TTT TTT GCA TCA CCA TAG TAT
841/281                             871/291                                901/301                                931/311
GAA ATT TTA AAA AAA AAA ATT TTT TTT ATA ATT TTT TTT TTT ATA ATT TTT TTT TTT TTT TTT TTT TTT TTT TTG ATT TTT TTT TTT TTT TTT TAA ATT TTT TTT TTT TTT CTT TTA ATT
961/321                             991/331                                1021/341                               1051/351
AAC ACA AAA AAA AAA AAA AAA ATC ATA TAC AAA TAA CAA TAT GGG TAA TAA ACA AGG TAA ATC CCC AAA TAA TAG CAA AGG AGG TAA AAA ATA CAG TAA GTA ATA TTA TAT AAT TCA CAT
                                                    M   G   N   K   Q   G   K   S   P   N   N   S   K   G   G   K   K   Y
1081/361                            1111/371                               1141/381                               1171/391
TTT ATT TAA TTT TAT TTA ATT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TAT TTA AAT TTT GTA CAT ACT AAT TTT TAT ATG AAT TTC TAA AAA TAG AAA TTG ATA ATG
                                                                                                                                                                                                I   D   N   D
1201/401                            1231/411                               1261/421                               1291/431
ATG TAG TCA AAC AAC TCC AAG AAT CAA CAA ATT GTA AGT AAT ATT ATA TAT ATT TAT AAA TAT TTA AAA TAA ATT AAT TAT TTA TTC TAT TTT ATT AAT TTC TTC TTT TTT GTT TTA TTT
  V   V   K   Q   L   Q   E   S   T   N
1321/441                            1351/451                               1381/461                               1411/471
TTT ATT TTA TTT TTT TTT TAT TAT TAA TTT TTT TTA TTA TTA ATT TTT TTT TTT TTT TAA TAT TTT TTT TTT TTT TAA TAT TAA AAG TTG ACA AGG TTG AAG CTA AAA AAT TAT ATG AAG
                                                                                                                                                                                                D   K   V   E   A   K   K   L   Y   E   V
1441/481                            1471/491                               1501/501                               1531/511
TTT TCT ATG ATC TTT CAA ATG GAG GTA AAG AAC CAT TAA ATA GGG ATA GAT TCA AAG AAG GTT TAA CCA AAT TAG AAT CAT GTG GTT TAA AGA ATC TTG ATA ATT CAC CAT TTG GTG ATC
  F   Y   D   L   S   N   G   G   K   E   P   L   N   R   D   R   F   K   E   G   L   T   K   L   E   S   C   G   L   K   N   L   D   N   S   P   F   G   D   R
1561/521                            1591/531                               1621/541                               1651/551
GTT TAT TCG ATT TAC TCG ATA CCA ATA AAG ATA ATA CTG TTG ATC TTC AAG AAT TTA TTA GTG GTT TAT CAA TTT TAT GTA AAG GTA CTG CCG AAG AAA AAC TCG AAT GTA ACT TTC AAT
  L   F   D   L   L   D   T   N   K   D   N   T   V   D   L   Q   E   F   I   S   G   L   S   I   L   C   K   G   T   A   E   E   K   L
1681/561                            1711/571                               1741/581                               1771/591
TTT ATT TAT TAT ATA TAT TTA TTT TTT TTT ATT TTT TTA TTT TTC CAA TAA TAA TAT ATA TAT ATA TAT ATT AAT ACT TCT TTT TTT TTA TTT TAT TTT ATT TTA TTA TTT TAT TTT TTA
1801/601                            1831/611                               1861/621                               1891/631
TTA TTT TTT TCA AAA AAA ATT TAG TAT CAT TTA AAG CAT ATG ATA TAG ATG GAA ATG GAT ATA TTA CAA AGA GTG AAT TAT CAC AAA TGT TTC AAC AAG CAT GGA TTT CAG GTT TCA AAG
                              S   F   K   A   Y   D   I   D   G   N   G   Y   I   T   K   S   E   L   S   Q   M   F   Q   Q   A   W   I   S   G   F   K   A
1921/641                            1951/651                               1981/661                               2011/671
CAT TAT CAT ATC AAA CTA ATG AAG AAG TCA ACA AGG ATG ATT TAA ATA ATT TTT CAG AGG AAA TGG CAC AAA TCT TTG CAG ATG GTG CTT TCT CAT CTT TAG ATG TTA ATG GTG ATG GTA
  L   S   Y   Q   T   N   E   E   V   N   K   D   D   L   N   N   F   S   E   E   M   A   Q   I   F   A   D   G   A   F   S   S   L   D   V   N   G   D   G   K
2041/681                            2071/691                               2101/701                               2131/711
AAT TAT CTT TCA ATG AAT TCA AAC AAT TCG CAA TGT CTC ATC CAA AAA TTA CTG CCA CAT TAA ATG GTT CAA AGA GAG ACG TGC CAA TTA CTT TTG ATT AAA TTA ATC ATA CAA CCA TTT
  L   S   F   N   E   F   K   Q   F   A   M   S   H   P   K   I   T   A   T   L   N   G   S   K   R   D   V   P   I   T   F   D   *
2161/721                            2191/731                               2221/741                               2251/751
CTT TAT TAA TAA AAA AAA TCA AAA AAA AAA AAG TAT ATA AAT AAA TTG TAA TTA ATA GAT TTT AAC TAA AAA AAA AAT AAA AAA AAA ACC CTC TTT TAT GGT TTA ATT TAT TTT GTT TTG
2281/761                            2311/771                               2341/781                               2371/791
GAA GAA AAA AAA AAA AAA AAA TAT CAT AAA TAC TTA GAA TAA AAA ATA AAA AAA AAA ATA AAA AAA AAA ATC ACA AAT TTT TTT TTT TTT TTT TTC TTT TTT GTT TTT TTT TTT TTT
2401/801                            2431/811                               2461/821                               2491/831
TTT TTT TAA TAT ATA TTA TTA TTT TTT TTT TTA TAT ATA CAA TTA CTA CTT TTT TTG TCA TTT TTT TTT ATT ATT TTT TTA TTT TAA AAA AAA TAC TCG AAT TTT AAA AGA TGA GTA
2521/841                            2551/851                               2581/861                               2611/871
AGT GGG GAA ATT TTA ATT TTG ACA TAT CAG CAG TTG GGA AGG TTA TTA AAA ATG TTG AAA AAT CAA TTG ACA AGG CAA TTG GTA TAC CAG ATC AAC CAG AAG GTA CTG AAA ACA ATG ATA
2641/881                            2671/891                               2701/901                               2731/911
ACA ATA CAT CAT TTT CTT CAT CTC CGG GTG GAG GTA CTA GTT TCT TTG GAT AAT CAT TTT CAA CAC CAA CAA ATT CTA CTT CAA ATA ATA CAC CAA TAA CAT CGA CAC CAA CAA AAT TAC
2761/921                            2791/931                               2821/941                               2851/951
AAA GTG GTC AAA CTC TCA CTC CAA CTC TTA CTC CTA TTC AAA GCA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ACA ATA ATA
2881/961                            2911/971                               2941/981                               2971/991
ATA ATA ATA GCA ATA AAC AAA CAA ACA ATA GTT CAA ATA ATA GTT CAA TAT TAA ATA TAA CAC CAA TAA AAC CAA AAA TAG AAC AAC AAT CCA TTC CCA CAC CTA AAT ATA GTG AAA ATA
3001/1001                           3031/1011                              3061/1021                              3091/1031
CAA GTG AGG AAA TTG GAT TAA AAA ATA AAG AGG ATA ATA AAG GTG AAG ATG AGG ATG AAG ATG AAG ATG AAG AAA AAC CAC TAT ATC AAA TAG ATG CTA ATT TAG ATT CAA AAT CTG AAA
3121/1041
ATA TTT TAA AAG AAG AGA AGG
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C

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/1                                  31/11                                  61/21                                  91/31
TTC AAA AAG TGT TTA AAA AAA AAA ATA AAA AAA TAA AAA AAT AAT TAA ATA TTT AAT AAA CAG TGA AAA CAA CAT CAT TTG ATT ATT ATA AAT TTT CTT AAT GTT ATA TTC TGT GTT TAT
121/41                                 151/51                                 181/61                                 211/71
TAT TTT TTT TTT TTA ATT TAT TTT TTT AAT TTG TTT TTT ATT TTT TTA TTT TTT TAT TTT TTT ATT TTT TAT TTT TTT TAT TTT TAC TCA ATT TAA ACA CAA CAA AAT GAT GTT GAG AAA
241/81                                 271/91                                 301/101                                331/111
AGT AGG TGG ATG CGG ACA TGA CCG CTT TTC AAT AGA AGA CCT TAA AGA ATC GGG CCC CTT TTT ATA CGA TGA TAA ATT ATT TGA CCA GGA TAG ATT CCC TGA GAT AAA TAG ACT CTG TAC
361/121                                391/131                                421/141                                451/151
AAA AGA ATG TAA AAA AAA ATG AAA GAA ATT TAT AGG ATA ACT TTC GAA GGA TAT CTC AAG GCA GTT AAT GAT TAC TAT GAT GAT TGT AAA ATA TTC AAA CGT CGC CCA AAC CCA CCA TAA
481/161                                511/171                                541/181                                571/191
TGT CAA TTG GCT GCC AGA CAT ACA ACG ATT GTT TAG AAT GGC GGA ATA GGG ATC CGG AGT ACC GTG ATC GTA TAT TAA AAA CTA TGA AAG CGG GAA TAC TTA ATG GTA AGC TAG TTA GAT
601/201                                631/211                                661/221                                691/231
TAT GTG ACG TGC CAA GGG GTG TAG ATG TAG AAA TTG AAA CAA CTG GGC TAA CCG ATT CAG AAG GAG AAA GTG AAT CAG AAG AAG AAG ATG AAG AAC AAG AAG AAG AAA ATG ATG AGA GTG
721/241                                751/251                                781/261                                811/271
ACA ATG AAT AAC TAC CAC TTT TAT ATC CGG TTA CCA TTA CCT ATA CAC ATT AAA CAC CGG AAA CCC CTT ACC GGT TAT CAG TTA CCG TAT CTA TCA CAA AAA CTA AAA AGC AAA AAA ATA
841/281                                871/291                                901/301                                931/311
AAC AGT TTA ACT TTT GTG AAC TAT AAT ATA AAT TTT TAT TAA TTA TTA TTG TTG ATT GTT GTT GGT GAT GGT GTT ATT ACT CAA GAA GAG AGT GAT TAT TAT TAT TCT CGT TCA AAA TCA
961/321                                991/331                                1021/341                               1051/351
ATA CAG TAT AAA AAT AAT AAA ATA TTA TAA TTT TAG AAT TAT GTT TTT GTA TAT TGT TCT CAG CAT TTT CTC CGA CAC AGG TAT TTA TTT AAT ATT AAA TTT TAT ATT TAT TAT TTT CAA
                                                     M   F   L   Y   I   V   L   S   I   F   S   D   T
1081/361                               1111/371                               1141/381                               1171/391
TAT AGG TTT GTA TTT TTT CTA ATT TCA CCA CTT TTT TGA AAC AAT AAA TGG TGA TAT AGA ATT TGA AAT TAA ATA TTT AAT AAA CAG TGA AAG CAA CAA CAA GTT GAT TTA TTA TTT TTT
1201/401                               1231/411                               1261/421                               1291/431
TAA TTT TAT TTT TAT TTT TAT TTT TTA TTT TAA TTT TTT TTT TTT TTT ATA TCG ATA TTT TTT TTT TTT TTT TTT TTT TTT TTG AAA AAA ATT TTA TGG TAA AAA GTA GTT TTG GGG
1321/441                               1351/451                               1381/461                               1411/471
AAT TTG TTA CAT CTA GGC AGA TCA AAC AAA TTG GGA ATG AAA ATA AAA TTA AAA TTA AAA AAA ATA AAA ATA AAA AAA AAA AAA AAT TAA ATA TAA TAT TCC TGT TTT TCG CAA CGT AGA
1441/481                               1471/491                               1501/501                               1531/511
TTT TTA ATT TTT ATT TTT ATT TTT ATT TTT ATT TTT AAT TTT ATT TTT TTA TTT TTT TTT TTT TTC TTT TTT TTT TTT TTT CTA TTT TTT TTT CTT TTT TTT GAA CAG TGT CAG TTT CAA
1561/521                               1591/531                               1621/541                               1651/551
ACC CAA TAT ATA TTT TTT TTT TTT CAT AAA TTT AAG GAT GAA GTC AAA ACG ATT ATT TTT TTT ATT ATG CCT GCT TCT GGT ATT CAT ATC TCA TTA TTC AGT ATC CAC TCT AGG TGA TGA
                                                                                                                                                                V   S   T   L   G   D   D
1681/561                               1711/571                               1741/581                               1771/591
TAG TGA TGA TTC GAA TAG TTT AAC TTT TCA TCC ACT ACC AAC AAC ACC ATC ATC ATC TTC AGC ACC CAC AAC AAC AAC ATC ATC AAC ATC AGC ACC CAC AAC AAC AAC AAC AAC CTC AAC
S   D   D   S   N   S   L   T   F   H   P   L   P   T   T   P   S   S   S   S   A   P   T   T   T   T   S   S   T   S   A   P   T   T   T   T   T   T   S   T
1801/601                               1831/611                               1861/621                               1891/631
ATC AGG AGG ATC ACA AGC AAC AGA ACC AGC AGA AAC ACC CAC AAC AAC AAC AAC AAC CTC AAC GTC AGG AGG ATC ACA AGC AAC AGA ACC AGC AGA AAC ATC CAC AAC AAC AAC AAC AAC
S   G   G   S   Q   A   T   E   P   A   E   T   P   T   T   T   T   T   S   T   S   G   G   S   Q   A   T   E   P   A   E   T   S   T   T   T   T   T   T
1921/641                               1951/651                               1981/661                               2011/671
AAC AAC AAT ACC AAC AAC AAC AAC AAC AAC AAC AAC AAT ACC AAC AAC AAC AAC AAC AAC AAT ACC AAC AAC AAC AAC AAC AAC AAT ACC AAC AAC AAC AAC AAC AAC AAT ACC AAC AAC
 T   T   I   P   T   T   T   T   T   T   T   T   I   P   T   T   T   T   T   T   I   P   T   T   T   T   T   T   I   P   T   T   T   T   T   T   I   P   T   T
2041/681                               2071/691                               2101/701                               2131/711
ATC AAC AAC ATC AAC AAC AGA ATC CAC AAA ACC TAC AAC ATC AGA TCC AAC GGG ACC TAC TCA TCT TGG AGA TGA AGA AGA TCC ATA CAG TGA TAA TTC AAG TAG GTT TTT TTT TTT TAA
S   T   T   S   T   T   E   S   T   K   P   T   T   S   D   P   T   G   P   T   H   L   G   D   E   E   D   P   Y   S   D   N   S
2161/721                               2191/731                               2221/741                               2251/751
AAA ATT TTA TTG TCT TTT TTG ATG TGT GTT TAT TAA ACT AAT TTA TTA ACA CTC CCA TAT ATA TAT AAT ATT ATA TAT ATG TAA AAA AAT ATA TTT AGT TTT CAA TAG TAC AAT TAT AGT
                                                                                                                                                                F   N   S   T   I   I   V
2281/761                               2311/771                               2341/781                               2371/791
ATA TGT AAT AGG CGG TGC ATG TGT AGT ATT TGT TTG TAT TAT ATT ATA TAA AAG ATT TAG AAC AGG TAG AAT TTC AAC AGC TCA AAA CAA TGC ATC ATA TGT TGC ATT AGA AAT GAA AGA
Y   V   I   G   G   A   C   V   V   F   V   C   I   I   L   Y   K   R   F   R   T   G   R   I   S   T   A   Q   N   N   A   S   Y   V   A   L   E   M   K   D
2401/801                               2431/811                               2461/821                               2491/831
TTA AAA AGA AAG AAA AAA ATA AAC AAG GAT CGA TCC AAT GTA AAT AAT ATA TTT AAT GAA ATA AAT AAA TAA ATA AAT AAA TAA AAA AAA AAA GAA CCA AAA ATT TCC TAT TTA TTA TTA
 *
2521/841                               2551/851                               2581/861                               2611/871
TTA TTA TTA TTA TTA TTT TTT TTT TTT TTT TAA TAG AAA ACT ATT TTT TAT AAT ACT ATA TTT TTT ATA ATA CTA TAT AGT TTA TTT TTT TTT TAT TTT TTT TAT TTT TAT TAA TTT AAA
2641/881                               2671/891                               2701/901                               2731/911
GAA AGA TTG GAA GCA AAA TGA CTT TTA ATA TAA TCA TAA CCT TTT TCT TGA TAA TCT TGT TTA GAG ATT GAA ACA TTT GAC CAA TTA TCT TGA TTA TCT AAA CAC CAT TTT CTA GCA CCT
2761/921                               2791/931                               2821/941                               2851/951
AAC CAA GCA TCC AAT TGA GAG TCT TTT GAT TTT AAG ATG GTT AAA GGT GAA TAT GGT TCA CGA ATT TGT TGA ATT TCA TAA TGA ATT CTA TCT TTA AAG TGT TTA GTA TTT ACA TTA CCA
2881/961                               2911/971                               2941/981                               2971/991
CCA GTG AGG AAG ATA TTC TCT GTT ACC AAT TTA CGT GTA TCC ACT GGC AAT TGG GAG AGA ATT GAA GAG ATA ATA GCC TCA ACC AAA CCC ATT TGA TCC ACA CCA ATG ATT GCT TTT GGT
3001/1001                              3031/1011                              3061/1021                              3091/1031
TGA AAT AAA GTT TCA GGA CAT TTA ATT CTT TCA ACG CCC AAT GAA ACT TGA AAA TCT TCA GCG GTA TGA TAT TCA CCT ATT GGG AAT TGA TCA TCA TGA CTA ACG ATG GCA CTA CTA ATA
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Figure 8 Genomic sequences of putative DdEB1 interactors. A EB1-6 **B** Calcineurin B **C** EB1-67. Sequences of the (predicted) open reading frames are shown along with 3' and 5' UTRs. Respective amino acid sequences are shown in single-letter code. Location of primer pairs used for amplification of the GFP constructs is marked by underlining the sequence. All sequences were taken from www.dictybase.org.

Three of these putative interactors were identified using DdEB1 as bait, one of them showing a homology to Calcineurin B, a subunit of a serine/threonine phosphatase involved in the immune response. For the other two, no homologous proteins could be found by blast search. Sequences of all three putative DdEB1 interactors are depicted in *Figure 8*.

The sequence of the first interactor, which turned out to be Calcineurin B (DictyBase ID DDB0191204), consists of 4 exons of a total of 678 bp, which corresponds to a

protein length of 225 aa. The molecular weight of this protein is 25.2 kDa. It contains a predicted calcium binding EF Hand motive (according to GeneDB (http://www.genedb.org)).

The second interactor was named EB1-6 (DictyBase ID number DDB0186613). It consists of one exon of 741 bp, which corresponds to a protein length of 247 aa and a calculated molecular weight of 26.7 kDa. Conducting a blast search, no hits were found.

The other putative DdEB1 interactor was named EB1-67 (DictyBase ID DDB0184032) and contains two exons of a total of 687 bp, which corresponds to a protein length of 229 aa and a mass of 23.9 kDa. Again, no hits were found conducting a blast search for this protein. According to GeneDB (http://www.genedb.org), it contains a signal peptide at the N-terminus and two putative transmembrane domains, one also at the very N-terminus and the other close to the C-terminus in the last fourth of the protein.

A

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ATT TTC AAA AAT ACA CCT TAT AAA TAA ATA TTT GTT TTG TAT AAT AAA AAA AAA AAA AAT AAA AAA AAA AAT AAA AAA ATT CAA ACT TTT AAA AAA TAT CTA TTT TTA AAT TTT ATT TTG
481/161                                 511/171                                 541/181                                 571/191
GTA ATA TAT ATA TAT ATA TTA TTT ATA TAT TTA AAA TAT CAT TTG ACA AAA TTT ATC ATC ACC CTG GGA ATT TAA GAT TAC TTT TTT TTT TTT TTT TTT TTT TTT TTT TTA TCA
601/201                                 631/211                                 661/221                                 691/231
TAA AAT TCA AAT CCA TAT TTA AAA TAA ATA TGA CAG AAT TTA AAA CAA GAT TTT ATG TCT ATT AAA ATA TTC TCA AAT TTT ATT TAA ACT TTG AGA ATA TTT TAT TTA AAC TTT GAG AAT
721/241                                 751/251                                 781/261                                 811/271
ATT TTA TTT AAA CTT TGA GAA TAT TTT ATT TAA ACT TTT AAA TAA TAA AAA GTT TTT TAT TCA AAG TTA AAT GTC TAT TTG CTT TTC AAA GAG TTT CAG TCA GAA TAT GGG ATC AAG GGG
841/281                                 871/291                                 901/301                                 931/311
AAT TTT TTG TGG GTT CCA ACC GAC CAG GAT TTT TGA TTT TCT ATT TTT TTT ATT TTA TTT TAT TTT TTT TTT TTA ATT TTA TTT TGG CAT TTT AGA ATT TTA TTT TTA ACT TTT TTT TAT
961/321                                 991/331                                 1021/341                                1051/351
TTT TTT TTT TTT TAT TTT ATT TTT AAA TTC AAG AAA GAA AAT GAG AAG TAT TTT ATC TTT ATT AAT TGT TGT AAT TTT ATC ATT TGT TGC TTT ATC AAA TGC AAC CGA TTG TGG AAA ATA
                                                    M   R   S   I   L   S   L   L   I   V
1081/361                                1111/371                                1141/381                                1171/391
TAA AAA TCC ATT GAC TTG TGC AAG TAA TTA TCG CTT ATT TAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA GAT CCA ATT TCA CTA ATA TTA TTT TAT TAA AAA TGA AAA GAT
1201/401                                1231/411                                1261/421                                1291/431
ATG GAA CCT CCT TGT GTA ATT ATT AAT TTA TAA ATA ATA AAA ATA ATA AAT AAT AAT TAA TAA TAT TAA TAG TAA TTA AAT AAC TAA TAC AAT ATC ACA TTT ATT GTT CTT AAT TTT AAA
1321/441                                1351/451                                1381/461                                1411/471
ATA GTT CTG GGA TGG TAA GGT TTG TAA AGA AAT TTA TCC AAA GAA CTG TGC AGA GAT GAC AGT TGA GGA TTG TGA AAG TAA CAA AGA AAA GGG ATG TTA TTT GGA GGG TGA AAA CTG TAT
     F   W   D   G   K   V   C   K   E   I   Y   P   K   N   C   A   E   M   T   V   E   D   C   E   S   N   K   E   K   G   C   Y   L   E   G   E   N   C   I
1441/481                                1471/491                                1501/501                                1531/511
TCT CTC AAA ATG TAA GTT TTT TAC TTT TTT TAT TTT TAA TTT ATT AAC CAG AAA TAT TAA TAT TTT TTT TTT TCA AAT ATT TAC CAA AAA AAA AAA AAA AAT AAA AAA AAA AAA TTT ATT
 L   S   K   C
1561/521                                1591/531                                1621/541                                1651/551
TAG TCC ATT GTT CAT CAA TCC ACA ATG AAT TTG ATT GCA CCA TCG AAA ATT GTG CAT GGA AAG ATG GAA AAT GTG AAA ATT AAA TTC TTT TAT TAA TTT TTT AAC AAT AAA TTA TTG ATG
1681/561                                1711/571                                1741/581                                1771/591
AAA TTT TAA TCA TTT AAT TAT TAT TTT ATT TAA TTT TCT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TGA TTA GAT TTA AAA ATA AAA ATT TAA AAT AAT ATT
1801/601                                1831/611                                1861/621                                1891/631
AGA TTT TTA AGT TTT AGT TTT TTA TTA GAA ATT TGG TTA ACT TTC TTC AAG GTA TTT TTA AAA AAA AAA AAA ATA AAA AAT AAA AAA TAA TAA CTT TTT TTA AAG GTA AAA ATA GAA
1921/641                                1951/651                                1981/661                                2011/671
GAA AGA AGG ATT TTT TTC TTT TTG AGA GGT TTT ATA ACT ATT AAA AAA ATA ATT ATA ATT TTT AAA AAT GAT ATG GTG TCA ACT TTA TGT TTG GCC TGA CAC TGT TTT ATA TTT TTT TAT
2041/681                                2071/691                                2101/701                                2131/711
GAA AAA AAT ATT TCA AGT AAA ATA ATT TTT TTG TTT TTT TTT TCA CAA CCA ACC GAA TTT AAT CTC CTG TTA TTT GCA CCA GAT CGT CAA AAA GTA AAA AAA AAA ATA TTA TTT TTC ATT
2161/721                                2191/731                                2221/741                                2251/751
GCG ATA TCC ATT TCC AAA AAA AAA AAA AAA TCA AAT GCT AAA CAA AGT TTT ATT TTA ATT TTT AAA CTT TTT TTT TTT ATT TTA CAT TTT TTG TAA ATG TTA AAA AAA AAA AAA AAC AAT
2281/761                                2311/771                                2341/781                                2371/791
CAG TTT AAG AAT TTG TTT TTT GTT TTG TAA GTA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA AAA ATA ATA ATA ATA ATA ATA ATA ATA ATA
                                          N   N   N   N   N   N   N   N   N   N   N   N   N   N   N   N   N   N   K   N   N   N   N   N   N   N   N   N   N
2401/801                                2431/811                                2461/821                                2491/831
ATA ATA ATA ATA ATA ATA ATA ATA ATA GTA ATT TAA AAT AAT TAA TAA AAA AAA ATT AAA CAA AAA AAA AAA AAA AAA AAA AAA ACA AAA AAA TCA AAT TGA AAT ATC TAT TAA TCC
  N   N   N   N   N   N   N   N   N
2521/841                                2551/851                                2581/861                                2611/871
AAA GCC AAA TTT TTC ATT GAA AAC CAA AAA TTT AAA TTC TTT TTT TTT CAG GTA ACC AAG AAA AAA AAA AAA AAA AAT ATA AAA AAA AAA ATT TTG TAA AAA AAA ATA TAA ATT AAA TAT
2641/881                                2671/891                                2701/901                                2731/911
TTA AAT CAA AGC AAA TTA AAT AAA TTA ATA TAT AAA TAA ATT AAA ATT TGA AAA TGA CAG GTA TGA TTT CAT ATA TAA TTT TTT TTT TTT TTT TTG AAA ATA ATT AAA ATA GTT AAA CTA
                                                                                                                                                        K   I   I   K   I   V   K   L
2761/921                                2791/931                                2821/941                                2851/951
ATA ATT TTA CAT CCA AAA GAA TAT ATA ATT GAA GCA GGA AAA TCA CAA AAA AAT GTT AAA ACC AAT CCA AAC CCA TCA GCG ACA TCA ATT AAC ATA GAA AAT TTG AGT AAA AGT GAT GAT
I   I   L   H   P   K   E   Y   I   I   E   A   G   K   S   Q   K   N   V   K   T   N   P   N   P   S   A   T   S   I   N   I   E   N   L   S   K   S   D   D
```

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2881/961                          2911/971                          2941/981                          2971/991
GGA AGA ATT AGA ATT ATT TCA ACA GTG AGT TTG AAA ATT TAT TTA ATT TTT TTT TTT TTT TTA AAA AAA TAT TAA AAA CTA ATC AAT ATT TAT TTA TTT ATT TTT TTT TTC TAA ATT AAA
G   R   I   R   I   I   S   T
3001/1001                         3031/1011                         3061/1021                         3091/1031
GTC ATT CCC TAT GGA AGA GGA TTT TAT TTC TCC TAT TTC CAA AAT AAC CAT TAA AAA AAA ATA TAA TAA AAA TCC AAT TGA AAT AAT GAA TTT AGG TAC AAC AGT TTT AAA AAT ATC AAT
3121/1041                         3151/1051                         3181/1061                         3211/1071
TGA ATA AAA AAT AAA AAA AAT AAA AAA AAA AGA AAA AAA ATG TTA TAT AAT AAA TAA AAC AAA AAT ATG TAA AAT TAA AAA AAA GGA CTG GAA AAA AAT AAA ATA GTT ATC CTT TGT ACA
                                                                                                                                                  L   S   F   V   Q
3241/1081                         3271/1091                         3301/1101                         3331/1111
ATA AAT ATT AAA TAA AAA AAA ATT TAA CAA ATG TTT AAA CTA GTT GTG TTT TTG TAT TTT ATG TTT TTT TTT TTT TTT ATT ATT TAT TTA TTT TGT TTA TTA TTT TCA TTT TTG AGA
 *
3361/1121                         3391/1131                         3421/1141                         3451/1151
AAC ATT TAA AAT GTT ATT AAT GTT ACC TCT ATC GTC AGT TAA AAT TGG TTT TTG GGG ATC ATA TTC CCA ATT TCC ATC GAT AAC AAA TTT GTA TTC GTA CCT ACC AGG CGC CAA GCG AAC
3481/1161                         3511/1171                         3541/1181                         3571/1191
AAC GAC TGA CCT TAT CTG TGT TTC GCT GGT ACT ATC TGA GCA TTT CTC TTC AAT TCT CGA CAA ATC TAT TGT TTG AGG CGG GGT ATT CAA TTG TGG ATC ATA GGT CAA GAG TAC TCT TTT
3601/1201                         3631/1211                         3661/1221                         3691/1231
ATC CCA ATT TAG GAA TGA TCC AGT CAA TTG AAT AAC ATG ACC ACT ATA TGG CCA TGT AAA AGT AAT GGG TAC TAA TAT TTC TGC ATC TTT TGA TAA TAA TAA TAA ATT ATT ACT ACT TAA
3721/1241                         3751/1251                         3781/1261                         3811/1271
ATT ATT AAT GTT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT GGT ATT AAT TAA ATT ATT ATT ATT AGA ACT ATT ATT GGC AAT ATT GTT AAT ATT ATT
3841/1281                         3871/1291                         3901/1301                         3931/1311
```

B

```
1/1                               31/11                             61/21                             91/31
AAG AAA ATG GAT CAT CAA GCA AAA AAG AAA GAA TTA GAT TTA TTA AAA CAA AAG TTT GGT TTG GAA TGA AAA TTT TCT ATT AAA AAA AAA AAA AAA AAA AAA AAA ATT AAT AAT AAT
121/41                            151/51                            181/61                            211/71
AAT AAT TAA AAA ACC TCG TTT GTA CAT AAT TAA AAA TAA TAT TTA TAA TAA AAA AAA ATA ATT ATA AAA AAA AAA ATA AAA AAA AAT AGT ATA TAA ATT AAG ATT CAT TGG CAT TTA TAA
241/81                            271/91                            301/101                           331/111
CTG GAT ATC TAA AGT TCA AAA TCT TTA ATA ATT GTT TAA ATT TAC GTT AAA GGA AAA AAA AAA AGA AAA AAA AAA AAA AGA GAA AAA AAA GAA GAA AAA AGA AAA AAA AAA AAT AAA
361/121                           391/131                           421/141                           451/151
AAA AAA AAT TAA GAA TAT TTA AGG ACA AAA CCT ATA TTT GAT AAT TTT TTA TTT TTT CAA TCT CAT TTT AAT ATA ATT ATT ATT TTA AAA TTT CCA ACA CAG CGG GTT ATT AGA TTT GGT
481/161                           511/171                           541/181                           571/191
AAA AAA AAA TGA AAG TAG TAC ATG GGA TAA TTT TTT TAT TTT TTT ATT TTT TTT TTT TGC TTA TAA TAA TTT TTT AAA ATC AAA TAA ATT AAA AAA AAA TTA AAA AAT AAA ATT TTT TAA
601/201                           631/211                           661/221                           691/231
AAT TTT TTT TTT AAT ATT ATT ACA TTT TAA TAA TAC CAA ACC AGC TAA TAA AAA ATA TTT ATT TAT TTA AAA TTT TTT TTT TTT TTA ATT AAA TTT TTT TTT TTT TTA ATT AAA TTT TTT
721/241                           751/251                           781/261                           811/271
TTA TTT TTT TTT TCA ATT TTA TTT TTT TTT CAA TTT TAT TTT TTT TTC AAT TTT GTT TTC TCA TTT TTT CAA TAA AAT TAA CTT TCT AAC ACA CCC ACA CAA ATA TTT TTT ATA TAT AAA
841/281                           871/291                           901/301                           931/311
CCT TTT TTT TTT TTT TTT TCT TTA ATA TAT TGC TAA TTT CCT AAA TAC ATA GTT TTT AAA ACA AAC ATT TAA TAT CAC TTT TTT TCT TAT AAC TTT TAT TTT TAT TTT TAT TTT TAT TTT
961/321                           991/331                           1021/341                          1051/351
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                                                  M   V   H   V   S   S   F   K   N   D   H   P   L
1081/361                          1111/371                          1141/381                          1171/391
ATA AAT TAC TAA CTA TTT TAA TTT TTT TTT GAA AAA AAT AAA ATA AAA TAA AAT AAA ATA AAA TAA AAC AAA ATA AAA TAA AAT AAA ATA AAA TAA ATA TAG ACA AAA GAA GAG AAG TAG
                                                                                                                                                  D   K   R   R   E   V   A
1201/401                          1231/411                          1261/421                          1291/431
CTG AAC GTA TTA GAT CAA AGT ATC TTG ATA GAA TTC CAG TTA TTG TAG AGA AAG CTC CAA GAT CAG ATG CAC CAG ACA TCG ATA AAA AGA AAT ACT TAG TAC CAG CTG ATA TTA CTG TTG
  E   R   I   R   S   K   Y   L   D   R   I   P   V   I   V   E   K   A   P   R   S   D   A   P   D   I   D   K   K   K   Y   L   V   P   A   D   I   T   V   G
1321/441                          1351/451                          1381/461                          1411/471
GTA AAT TTG TTT ATG AAA TTA GAA AGC ATA TGA CCA AAG TTA GTG CTG AAA AAG CCA TTT ATT TGT TTG TTA ATA ATA CTA TCC CAC CAA CTG CTG CTT TAA TTT CTC AAA TTT ATG AAC
  K   F   V   Y   E   I   R   K   H   M   T   K   V   S   A   E   K   A   I   Y   L   F   V   N   N   T   I   P   P   T   A   A   L   I   S   Q   I   Y   E   R
1441/481                          1471/491                          1501/501                          1531/511
GTT ATA AGG ATG AAG ATG GAT TTT TAT ACA TTA CTT ACA GGT ATG CAT TTC TTT TTT TAT CTT TTT TTT ATT TAT TTA CAA ATC TTA TAA TTC TAA CAT ATT ATT CTT AAT TTT ACT ATT
  Y   K   D   E   D   G   F   L   Y   I   T   Y
1561/521                          1591/531                          1621/541                          1651/551
TTT TAG TGG TGA AAA TAC TTT TGG TAG TGA TTT ATA ACC TAT TAT AAT CCA TGA ATG TAT TCA TGC ACT CTG AGA GAA AGA AGA CCA CCA AAT GTA TAT AGA TCC AAA CAA TAA TAG TTT
    S   G   E   N   T   F   G   S   D   L   *
1681/561                          1711/571                          1741/581                          1771/591
GCC AAA TGA ATG GAA ACT TTA ATC TCT TTT AGT TAT GAT ACT TAC ACA ATT GTA AAT CTT CTT TTT TCA ATT TCT AAA AAC AAA AAA AAA AAA ACA AAA AAA AAA AAA ATA AAC AAA CAA
1801/601                          1831/611                          1861/621                          1891/631
AAA AAA AAA AAC AAA ACA AAA ATA ACT TTT CTA TTG TTT TTT TTT TTT ATA CAT ATA TGT TTA AAC CAA AAT AAT CTG GTT TAT TTT TAT TTA TTA TTG TTA TAG TTT CCT ATT TTT AAA
1921/641                          1951/651                          1981/661                          2011/671
AAA TTA TAT AAA GTA AAA ATA AAA TTA TAA AAA AAA AAA GGT TAG TTT TTT GAT TTG GTA ACA ATG TTT TTA ATT TAG TTA CAA TGT TTT TTA TTA CTT ACC AAA ATA TGG TTG GCC TGA
2041/681                          2071/691                          2101/701                          2131/711
TTG TTC AAT TAT CCT TCT TTG TTG TTC AAT AAT ACC TAA ACC CTC CTG TTT TTG TTG ATT ATA ATA CTG TGG TGA CTG GTA ATT TAA ATG TGC AGG AGG TGA TTG TGT TTG TTG TTG GGT
2161/721                          2191/731                          2221/741                          2251/751
TGA TCT TTG TAA ATA ATA CTG TGG ATC GAA TGG ATA TGA CTG ATT TTG TTG GTT TTG ATT ATG TTG GCT TTG ATT ATG TTG TTG GTT TTG ATT ATG TTG TTG GTT TTG ATT ATG TTG TTG
2281/761                          2311/771                          2341/781                          2371/791
ATA TTG TTG TTG TTG CTG TTG TTG CTG TTG GTG TTG GTG TTG TTG GTG CTG ATG ATG ATG TTG ATG TTG TTG GAA TTG TTG TTG AAT TTG TTG GTG AAT TTG TGG TTG TTG CAT TGG ATT
2401/801                          2431/811                          2461/821                          2491/831
GTG TTG TTG TTG TTG TTG CTG TTG TTG TTG TTG TTG TTG TTG TTG TAG TTG TTG TTG TTG TTG TAA TTG TTG AAA TTG TTG CTG TAT TTG TTG TTG AAT TAG TGG TTG TTG TGT TGG TGA
2521/841                          2551/851                          2581/861
ACG ATT TTG GTT GAG TTG TGA TTG TTG ATT ATT GGA GTT ACC ACC TTG TTG ACT GTC TAC AAT ATT CAT TAA GGT TG
```

Figure 9 Genomic sequences of putative N-terminal DdCP224 interactors. A DdCP n-term-27 **B** Atg8. Sequences of the (predicted) open reading frames are shown along with 3' and 5' UTRs. Respective amino acid sequences are shown in single-letter code. Location of the primer pairs used for amplification of the GFP constructs is marked by underlining the sequence. All sequences were taken from www.dictybase.org.

Two putative interactors were identified using the N-terminal part of DdCP224 as a bait (sequences are shown in *Figure 9*), one has been previously characterized as atg8 (Otto *et al.*, 2003) and the other one again shows no homology to other known

proteins. It was named DdCPn-term-27 (DictyBase Id DDB0216686) and has a length of 345 bp distributed over 4 exons and a protein length of 115 aa. The calculated molecular weight is about 13 kDa. GeneDB (http://www.genedb.org) identifies one possible signal peptide and transmembrane domain at the very N-terminus of the protein, although these were not confirmed by an analysis with PSORT (http://nibb.ac.jp), which calculates a nuclear localization for this protein.
Interestingly, Atg8, which is known to be involved in autophagy and used as a marker for autophagosomes (Otto *et al.*, 2004), also shows homology to MAPs (microtubule associated proteins) in various species (*Figure 10*) (Ketelaar *et al.*, 2004). It has three exons of a total of 369 bp and a protein length of 22 aa. The molecular weight is 14.1 kDa.

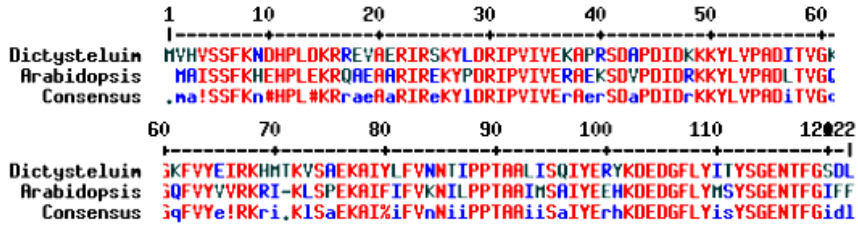

Figure 10 Homologies of DdAtg8 to *Arabidopsis thaliana* Atg8. Identities: 70 %, Positives: 83 %. Alignment was generated using Multalin Version 5.4.1 (Corpet, 1988). Symbol comparison table: blosum62, Gap weight: 12, Gap length weight: 2, Consensus levels: high = 90 % (shown in red) low = 50 % (shown in blue). Consensus symbols: ! is anyone of IV; $ is anyone of LM; % is anyone of FY; # is anyone of NDQEBZ.

Unfortunately no putative interactors could be identified using the C-terminal part of DdCP224 in this screen.

1.4 Cloning of putative interactors

To shed more light on their putative function and verify the interactions, all putative interactors were cloned by PCR amplification from library plasmids, RT PCR or amplified from cDNA (λ ZAPII phage library, (Gräf *et al.*, 2000)) and GFP-fusions of all proteins were expressed in *Dictyostelium discoideum* cells. EB1-6 was amplified from cDNA, whereas Calcineurin B, EB1-67 and DdCP n-term-27 were amplified form their respective yeast library plasmids. Atg8 was amplified by RT-PCR. All PCR primers were modified with Sal I and BamH I restriction sites and PCR products were cloned into vector pA6PGFPV18 or pV18A6PGFP-L-SSEB.

1.5 Investigation of subcellular localization by GFP fusion

All plasmids containing the DNA for the expression of the GFP-fusion proteins of the putative interactors were transformed into *Dictyostelium discoideum* AX2 cells and bona fide transformants were selected by their GFP fluorescence as well as by Western blot analysis (data not shown). For Calcineurin B and EB1-67 no specific localization of the GFP fusion protein could be shown.

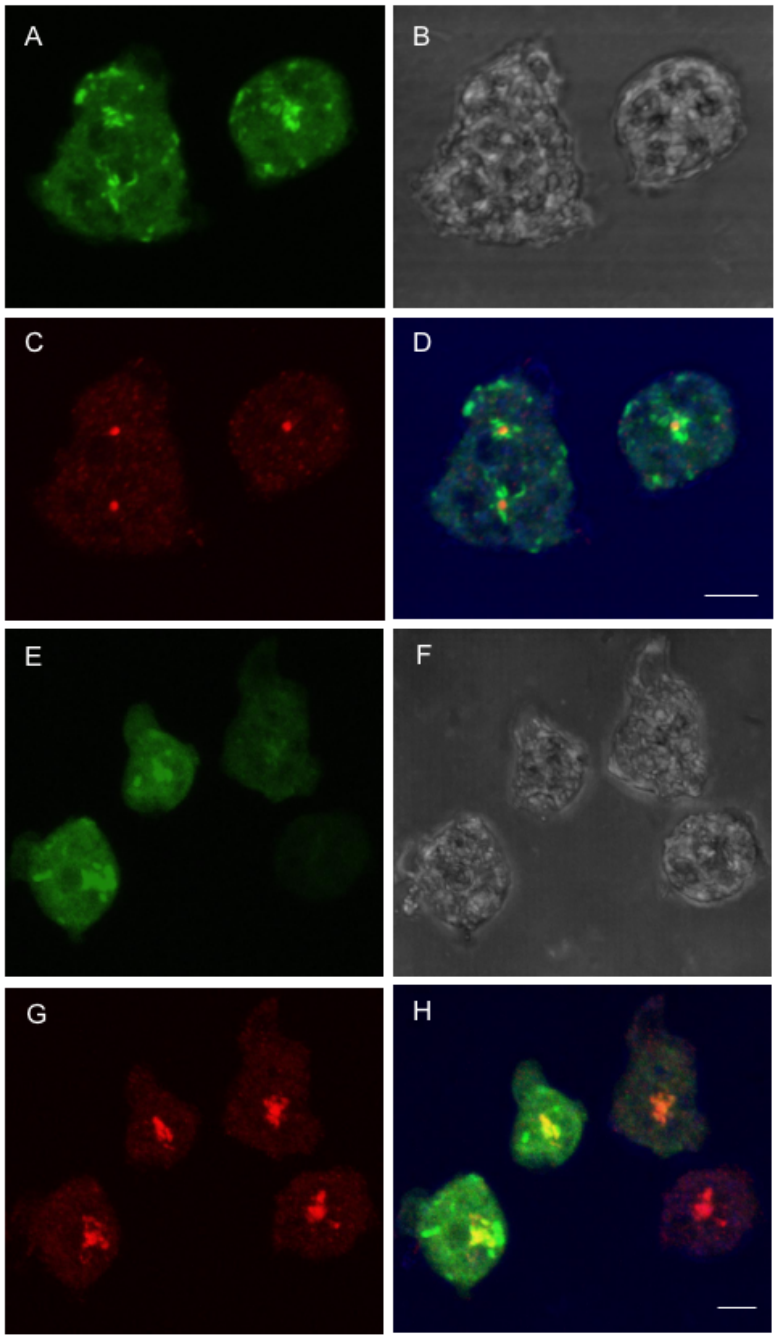

Figure 11 Subcellular localization of GFP-EB1-6 fusion. A GFP fluorescence **B** Phase contrast of the same cells. **C** Immunofluorescence microscopy with a DdCP224 antibody. **D** Merged images A-C. **E** GFP fluorescence **F** Phase contrast of the same cells. **G** Immunofluorescence microscopy with a Comitin antibody. **H** Merged images E-G. Bar 5 µm.

As can be seen in *Figure 11*, EB1-6 clearly localized to the perinuclear region. A double stain with a Comitin antibody (Weiner *et al.*, 1993) reveals the localization of EB1-6 to the Golgi apparatus.

DdCP n-term-27 seems to possess a distinct cellular localization concentrated in the perinuclear region, as can be seen in *Figure 12*, but it could not be related to the centrosome or the microtubule plus end, where a colocalization could indicate a physiologically relevant interaction with DdCP224.

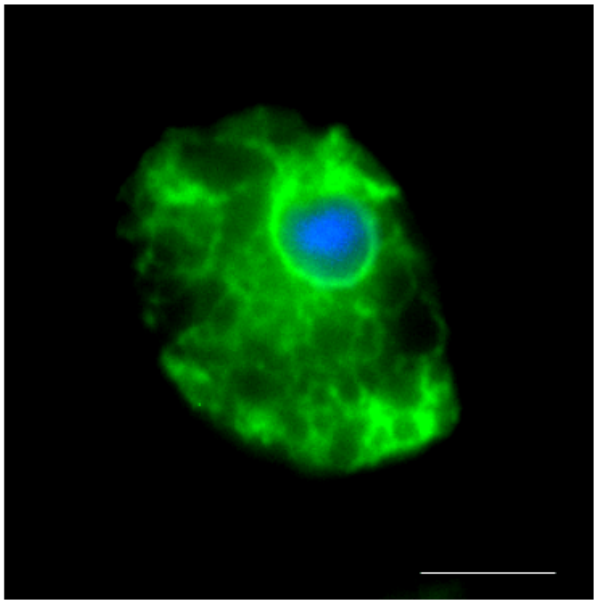

Figure 12 Subcellular localization of GFP-DdCPn-term-27 fusion. Nuclei were stained with DAPI and are shown in blue. Bar 5 µm.

The localization of the GFP- Atg8 fusion protein shown in *Figure 13* matches the one described by Otto et. al who claims Atg8 to localize to autophagosomes and isolation membranes (Otto *et al.*, 2003) and uses it as a marker for autophagosomes (Otto *et al.*, 2004).

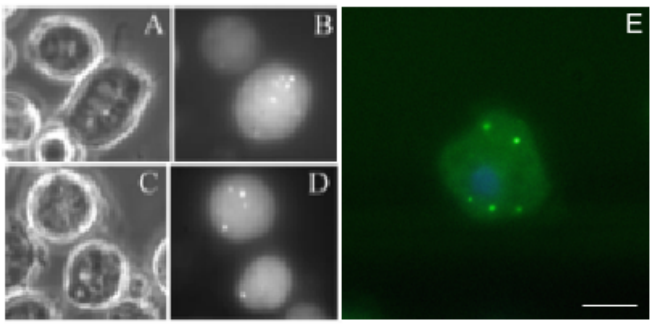

Figure 13 Subcellular localization of GFP-Atg8 fusion. A phase image **B** GFP fluorescence **C** Phase image **D** GFP fluorescence. **A+B** cells grown in full medium **C+D** cells starving **E** strain KK1, expressing GFP-Atg8. GFP fluorescence. Nucleus stained with DAPI. **A-D** taken from (Otto *et al.*, 2003). Bar 5 µm.

1.6 Attempted verification by co-immunoprecipitation and GST pulldown

For all putative interactors identified using the yeast two-hybrid system several independent attempts of co-immunoprecipitation as well as GST pulldowns were undertaken. All PCR products described in 1.4 were modified with BamH I and Sal I restriction sites and cloned into vector pGEX4T3. Unfortunately, none of the coimmunoprecipitation and GST pulldown experiments yielded a positive result even after numerous attempts (data not shown).

Since all the putative interactions found could not be verified by other means, another strategy to look for interactors of DdEB1 and DdCP224 had to be implemented.

2 Screening for interactors by Tandem Affinity Purification

To overcome problems resulting from the heterogeneous expression of the *Dictyostelium discoideum* proteins, it was decided to adapt the Tandem affinity purification (TAP) method to *Dictyostelium discoideum*.

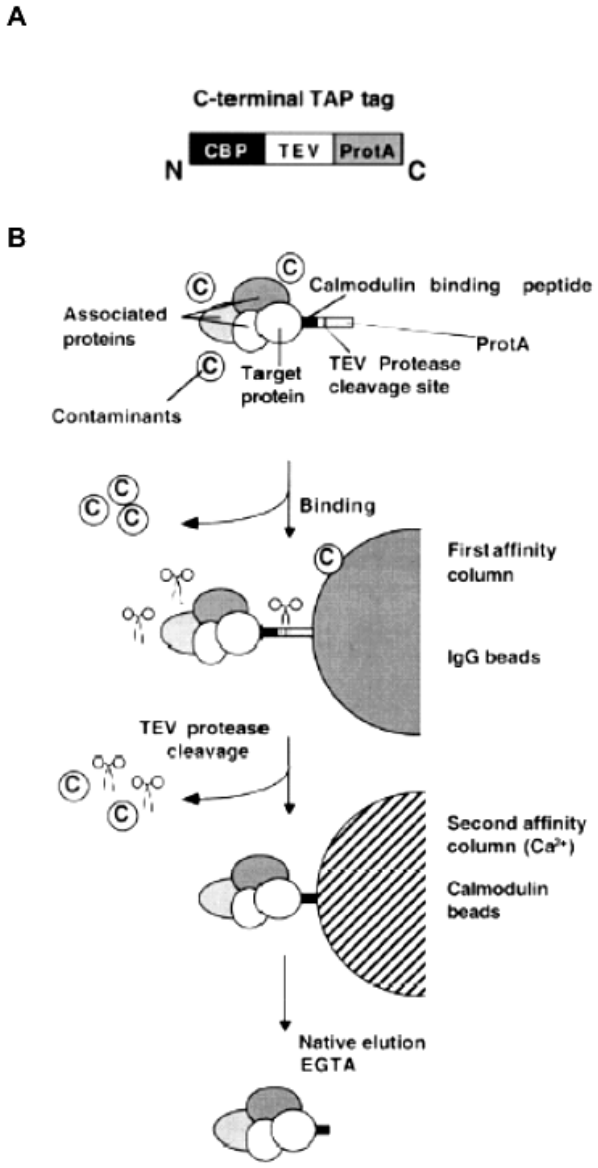

Figure 14 Overview of the TAP procedure. A The TAP tag was fused C-terminally to the proteins of interest. The TAP-tag consists of IgG binding units of Staphylococcus aurous protein A and a calmodulin binding peptide (CBP) with a cleavage site for Tobacco Etch Virus (TEV) protease between them. **B** The TAP procedure here shown for a C-terminal tag: the first step utilizes the binding of the ProtA moiety in the tag to matrix-bound IgG. The complex is released from the beads by cleavage of a seven-amino acid residue recognition sequence located between the two tags by TEV protease. The complex is then immobilized via the calcium-dependent binding of the calmodulin-binding peptide to calmodulin-coated beads and released by the addition of EGTA. Overview modified from Puig et al., 2001 .

TAP is a chromatographic method that was originally developed in yeast (Puig *et al.*, 2001; Rigaut *et al.*, 1999), where it had several successful applications in elucidating novel protein interactions in this organism (Gavin *et al.*, 2002; Gingras *et al.*, 2005; Shevchenko *et al.*, 2002), as well as in the closely related fission yeast (Gould *et al.*, 2004; Horn *et al.*, 2005; Tasto *et al.*, 2001). For mammalian cell lines several successful approaches of modified tandem affinity purification have been reported recently (Drakas *et al.*, 2005; Li *et al.*, 2004) and the method was also successfully used in *C. elegans* and *Drosophila* (Rubio *et al.*, 2005; Veraksa *et al.*, 2005). Employment of such a proteomics approach in a search for interactors was greatly facilitated by the completion of the *Dictyostelium* genome project (Eichinger *et al.*, 2005).

The TAP-tag described in *Figure 14* is a protein tag consisting of a calmodulin-binding protein and the IgG-binding domains of protein A, which are separated by a cleavage site for the highly specific TEV-protease. The tagged protein of interest and its associated proteins within a cytosolic extract are purified by two subsequent affinity purification steps using IgG-beads and calmodulin-beads. In the first step, the protein complex is bound to IgG-beads via the Protein A part of the tag at the very C-terminus. The elution from the first column is conducted via TEV protease cleavage (Senger *et al.*, 1998), which is highly specific and thus allows elution of the protein-of-interest. Unspecifically bound cytosolic proteins containing no TEV-protease recognition site remain on the column. For further purification the eluate is bound to calmodulin beads and EGTA is used to elute highly purified protein complexes. The latter are then subjected to gel electrophoresis followed by mass spectrometrical analysis of individual protein bands.

2.1 Generation of a *Dictyostelium discoideum* TAP-tag expression vector

In order to perform Tandem affinity purification in *Dictyostelium discoideum*, an expression vector for this organism had to be generated. Therefore a *Dictyostelium* C-terminal TAP-tag vector containing a blasticidin resistance cassette was constructed through the addition of a BamH I/Nsi I fragment from the yeast TAP-tag vector pBS1539 (Puig *et al.*, 2001) to p1ABsr8 (Gräf *et al.*, 2000). This vector was named pKK4 and a map of it is shown in *Figure 15*. To insert the sequence of interest, restriction sites for Hind III, Kpn I or BamH I can be used.

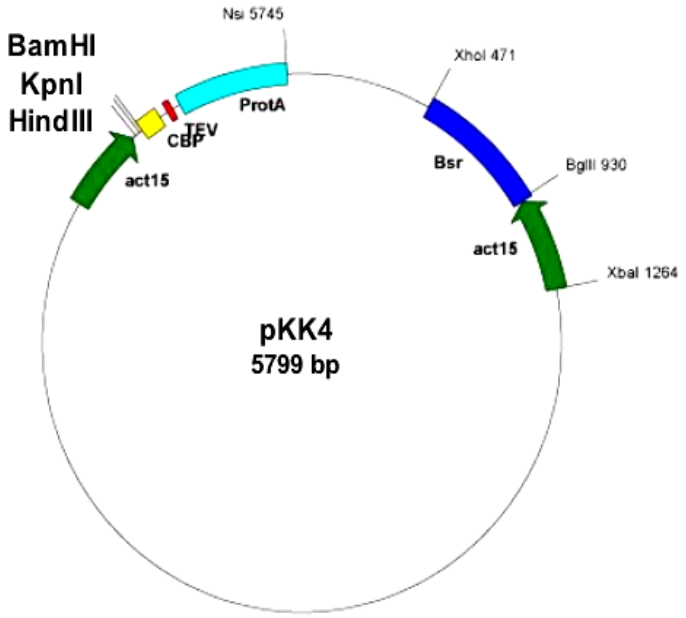

Figure 15 Plasmid map of the *Dictyostelium* TAP-tag expression vector pKK4. A multiple cloning site containing Hind III, Kpn I and BamH I sites is inserted between actin 15 promotor and the C-terminal TAP-tag. The Bsr cassette serves selection of transformants in *Dictyostelium*.

2.2 Cloning of baits into TAP vector

The vector was used to express TAP-tagged versions of DdEB1 and the N-terminal or C-terminal parts of DdCP224, respectively in *Dictyostelium discoideum*.
Splitting DdCP224 into these two parts allowed better handling due to the reduction of its large size and further enabled to distinguish between interactors of the N- and the C-terminus. All plasmids are shown in *Figure 16* and described in detail in 3.18 (materials and methods).

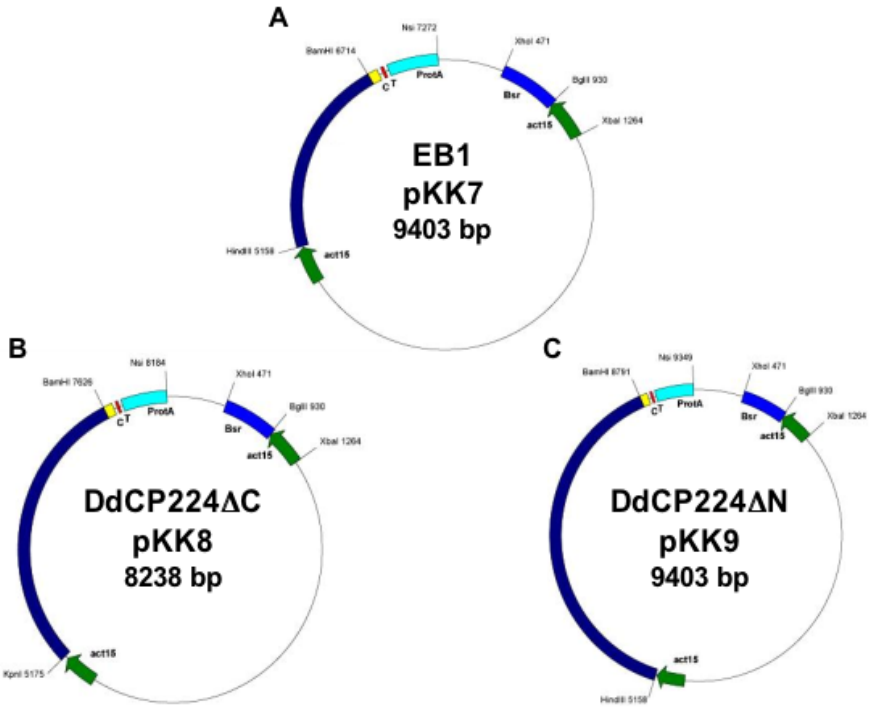

Figure 16 Map of TAP expression plasmids used in this study. A pKK7 shows a plasmid for the expression of EB1TAP. **B** pKK8 is a plasmid for the expression of the N-terminal 813 amino acids of DdCP224 with the tag. **C** for the expression of the C-terminal part of DdCP224 with the tag, pKK9 was created.

2.3 Expression and cleavability of constructs

Before conducting Tandem affinity purification, the plasmids were transformed into *Dictyostelium discoideum* AX2 cells to create the strains KK7, KK8 and KK9, which each contain the respective plasmids, pKK7,8 or 9, and should therefore express the tagged proteins. To test for the expression of these constructs, Western blot analysis was performed using protein extracts of these strains. As a control, protein extracts from wild type AX2 cells were blotted next to the mutant strains. Blots were incubated with appropriate antibodies against the protein-of-interest encoded by the TAP-expression vectors.

As can be seen in *Figure 17*, all three strains expressed the tagged proteins along with the endogenous proteins in the expected size. Another important prerequisite for a successful purification is the accessibility and cleavability of the TEV-protease cleavage site in the constructs to be used. To test for that, protein extracts of strains KK7-9 were incubated with TEV protease under conditions similar to the actual purification and subsequently analyzed by Western blot (*Figure 17*). As expected, after removal of the Protein A containing part of the tag by TEV cleavage, a clear reduction in size of the TAP-tagged fusion proteins was detected.

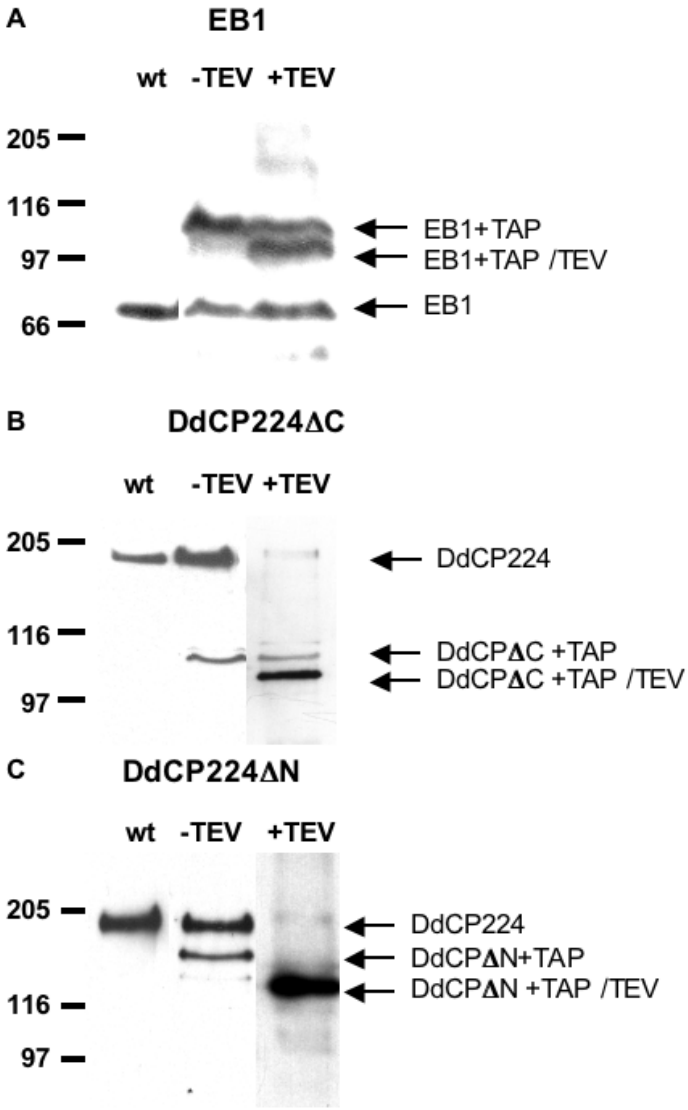

Figure 17 All baits are expressed and cleavable. A Expression of DdEB1 and DdEB1TAP detected with a DdEB1-antibody. Native DdEB1 can be found at about 70 kDa, addition of the TAP-tag increases the size to about 110 kDa. Removal of the protein A part of the TAP-tag slightly reduces the size of the tagged protein. **B** Expression of DdCP224 and DdCP224ΔC-TAP detected with an antibody against the N-terminus of DdCP224 (anti-DdCP-Hind III). Native DdCP224 can be found at about 200 kDa, the N-terminus with addition of the TAP-tag can be seen at about 105 kDa. Removal of the protein A part of the TAP-tag slightly reduces the size of the tagged protein. **C** Expression of DdCP224 and DdCP224ΔN-TAP detected with a monoclonal antibody against the C-terminus of DdCP224 (2/165). Native DdCP224 migrates with an apparent molecular mass of approximately 200 kDa; its TAP-tagged C-terminus can be detected at approx. 130 kDa. Removal of the protein A part of the TAP-tag slightly reduces the size of the tagged protein. For all blots protein extracts prepared as described for tandem affinity purification were used. For TEV cleavage, 500 µl of cell extract were incubated with 10 units of TEV protease at 16°C for 2 h. The protease inhibitor cocktail present in the extracts does not significantly inhibit TEV protease.

2.4 Localization of tagged proteins

For a successful identification of members of the complexes containing DdEB1 and DdCP224 both at the centrosome as well as at the microtubule plus ends, the tagged proteins need to be physiologically functional. Since no knock-out strains with an easily recognizable phenotype are available for all bait proteins, the localization of the fusion proteins was analyzed. Functionality of the fusion proteins was then assumed based on correct localization.

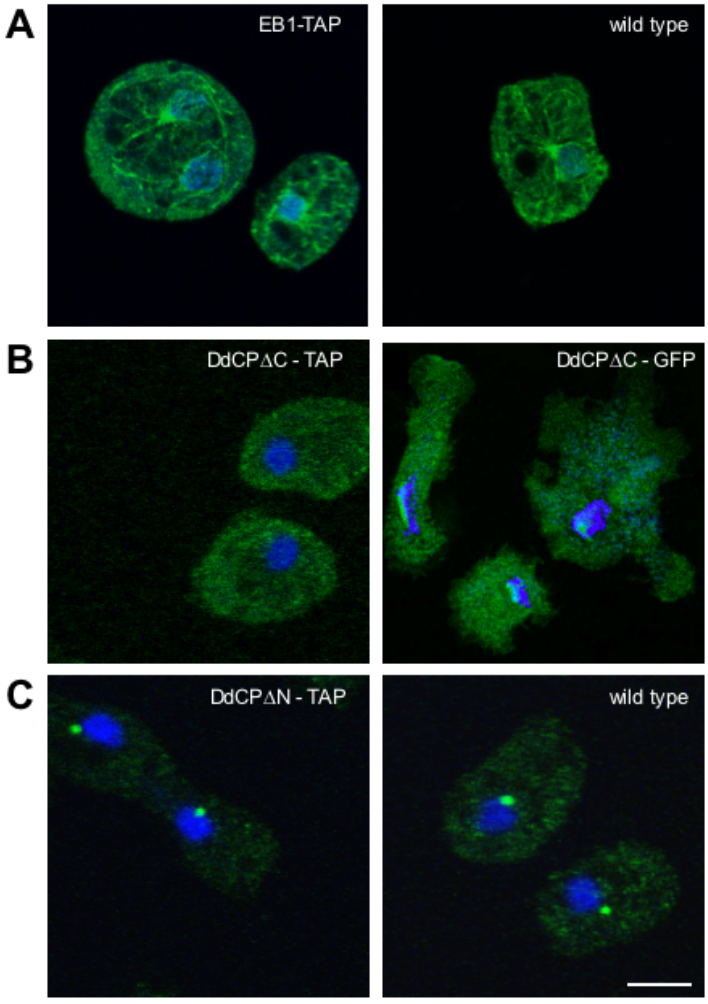

Figure 18 Unaltered localizations of tagged proteins. A Immune fluorescence microscopy with an anti-DdEB1 Antibody. DdEB1-TAP was transformed into a ΔEB1 strain. Localization of tagged DdEB1 was unchanged compared to the endogenous protein in wild type cells. **B** Cells of a DdCP224ΔC-TAP strain were stained with a TAP-tag antibody. For comparison, cells of a DdCP224ΔC-GFP strain are shown (taken from (Hestermann & Gräf, 2004)). Localization of both signals was similar, i.e. centrosomal localization is almost lost in absence of the C-terminus. **C** Cells of a DdCP224ΔN-TAP strain were stained with an anti-TAP-tag antibody. For comparison, wild type cells were stained with a monoclonal antibody against the C-terminus of DdCP224 (2/165). Localization of both signals was similar. In all images nuclei were stained with TOPRO3 (Molecular Probes) and are shown in blue. Bar 2.5 µm.

In case of DdEB1, an available DdEB1 knockout strain (Rehberg & Gräf, 2002) was used as a target strain. As this strain already contains a blasticidin resistance cassette an DdEB1-TAP vector with G418 resistance was created (pKK16).
Similar to AX2 wild type control cells, DdEB1 localizes along microtubules, at microtubule tips and at the centrosome *(Figure 18* A). For DdCP224 no knockout strains are available since the complete depletion of DdCP224 seems to be lethal (Gräf, 2001a). In this case, an antiTAP-tag antibody was used in order to distinguish between (i) the wild type protein and (ii) the tagged parts. The wild type cells used as controls were stained with antibodies against the appropriate parts of DdCP224. To be able to assess the diffuse localization of the N-terminal part of DdCP224, the staining pattern of the tagged protein was compared to a strain expressing a GFP tagged version of this part of DdCP224 (Hestermann & Gräf, 2004). The TAP-tagged DdCPΔN and DdCPΔC fragments showed the expected localization pattern. It is known from the GFP-tagged variants that the C-terminal part of DdCP224 can be detected only at the centrosome and not at microtubule-plus-ends and the N-terminal part loses the centrosomal localization and is found in the cytosol (Gräf *et al.*, 2000; Hestermann & Gräf, 2004). As displayed in *Figure 18*, localization of all three constructs was not altered by addition of the tag.

2.5 Adaptation of purification to *Dictyostelium discoideum*

In order to perform Tandem affinity purification in *Dictyostelium discoideum*, a gentle method to prepare cell extracts had to be used to ensure that most of the protein interactions and complexes would not be destroyed during the procedure.
Therefore cells were lysed by repeatedly passing them through a 5 µm Nucleopore filter and kept in the buffer used for the first affinity step as described in 4.11 (materials and methods). For the actual purification a protocol modified from the original yeast protocol (Rigaut *et al.*, 1999) was used (see materials and methods 4.11). Attempts to lower the pH of the used solutions to 6.8 to resemble the conditions present in the cytosol of *Dictyostelium discoideum* more closely or omitting the detergent NP40 did not yield better results for the proteins tested in this study as can be seen in *Figure 19* but might be useful for the investigation of other protein complexes.

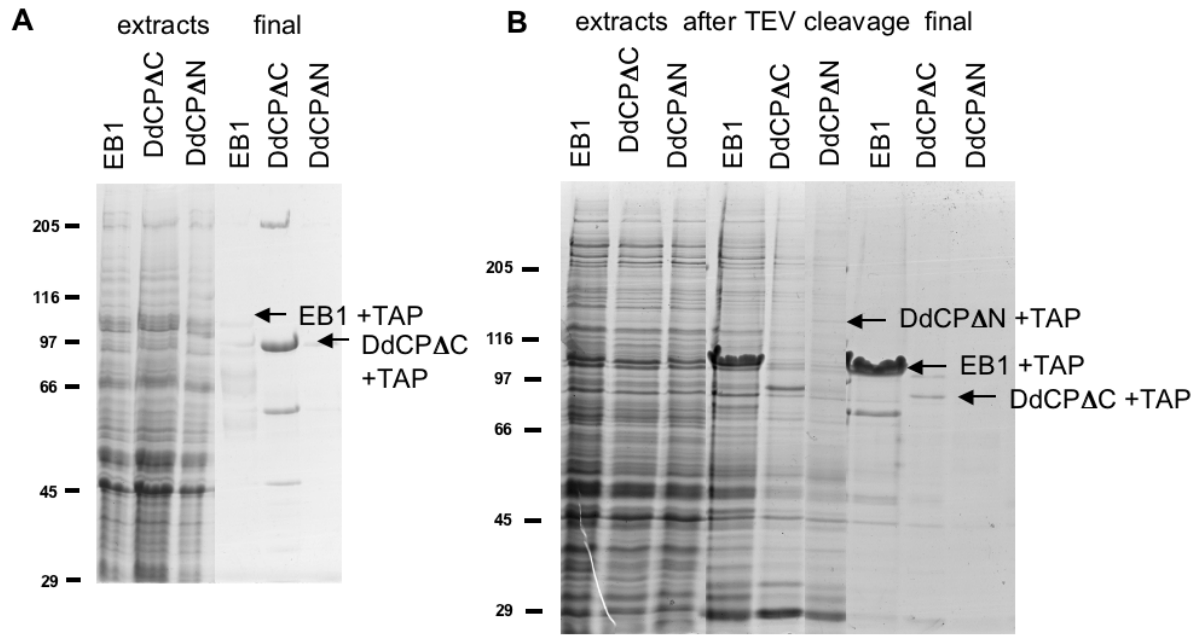

Figure 19 Varying conditions of Tandem affinity purification. A TAP performed at pH 6.8. 10 ml of cytosolic extracts (about 6 µg protein/µl) of wild type strain AX2 as well as all three bait strains were subjected to Tandem affinity purification. Samples were subjected to SDS page and silver stained. Arrows label bands representing the tagged proteins present in visible amounts. **B** TAP performed omitting NP40. 10 ml of cytosolic extracts (about 6 µg protein/µl) of wild type strain AX2 as well as all three bait strains were subjected to Tandem affinity purification. Samples were subjected to SDS page and silver stained. After TEV cleavage (2nd set of lanes), the tagged proteins can be detected. Arrows label bands representing the tagged proteins.

2.6 Enrichment of fusion proteins

After the first attempts of Tandem affinity purification, aliquots were taken from (i) the cytosolic extracts, (ii) the supernatant of the IgG beads after TEV cleavage (1/10 of the total volume) and (iii) of the final eluate and subjected to SDS-gel electrophoresis followed by silver staining. One of these gels is shown in *Figure 20*. It clearly reveals that already after the first purification step (2nd set of lanes) all tagged proteins are greatly enriched compared to the cytosolic extracts. The second purification step via the calmodulin column reduces the amount of unspecific bands also present in the control and enhances the level of enrichment of the fusion proteins. Along with bands exhibiting the size of the tagged proteins, also several other bands, both smaller and larger than the tagged proteins are visible and partially specific for the different constructs used. Therefore, the methods proved to be suitable to identify members of the complexes-of-interest. The final eluate was TCA precipitated and the pellet was send to our cooperation partner Dr. Yvonne Reinders (laboratory of Dr. Albert Sickmann at the Rudolph-Virchow-Center for Experimental Biomedicine in Würzburg, Germany) for further analysis.

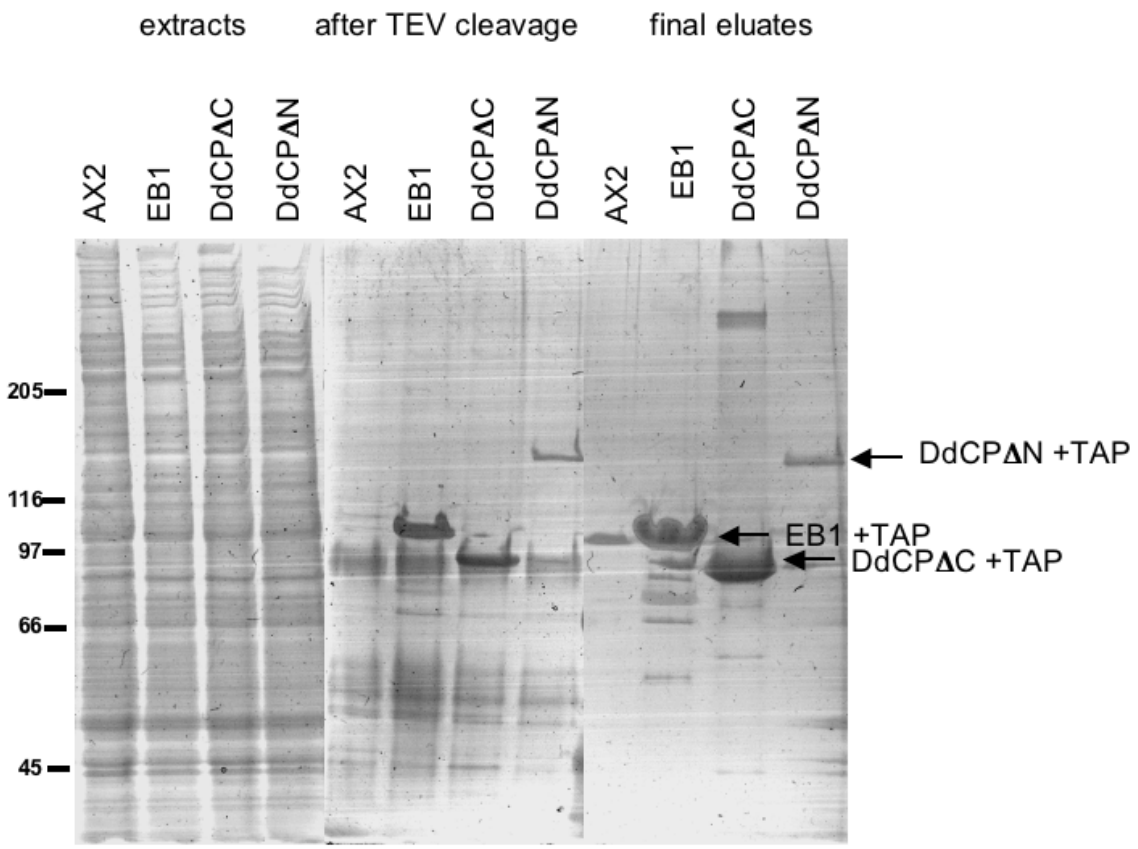

Figure 20 Enrichment of tagged proteins. 10 ml of cytosolic extracts (about 6 µg protein/µl) of wild type strain AX2 as well as all three bait strains were subjected to Tandem affinity purification. Samples were subjected to SDS page and silver stained. After TEV cleavage (2nd set of lanes), enrichment of the tagged proteins can already be detected. Further purification via the second column results in an even purer preparation of the complexes to be investigated. Arrows label bands representing the tagged proteins.

2.7 Analysis of putative interactors

Subjection of individual protein bands cut out of SDS-gels to LC-mass spectrometrical analysis (see 4.11, materials and methods) showed that the faint bands visible in controls of mock purifications of the wild type strain AX2 represented mainly ribosomal proteins as well as discoidin and actin. When strain KK4 (the TAP-tag without a bait attached to it) was used as a control, calmodulin appeared in addition to the previously found contaminants. Calmodulin was also present in all analyses performed with strains expressing tagged proteins-of-interest. This is most likely due to the presence of the calmodulin binding peptide in the tag.

EB1	DictyBase ID	
	DDB0189914	DdCP224
	DDB0215963	Imidazoleglycerol-phosphate synthase
	DDB0185122	Guanine nucleotide-binding protein
	DDB0191168	Heat shock protein Hsp70Bb
		proteins involved with the actin cytoskeleton:
	DDB0191243	F-actin capping protein alpha subunit
	DDB0216426	Rubredoxin reductase
	DDB0214935	p34 arp2/3 complex 34 kDa subunit
		metabolic proteins (e.g. glucose-6-phosphate dehydrogenase)
DdCP∆C		
	DDB0191160	EB1
	DDB0191169	Tubulin
	DDB0215409	GTP-binding nuclear protein RAN/TC4
	DDB0186513	G protein-binding protein CRFG (nucleolar)
	DDB0185297	Elongation factor 1-γ
	DDB0191174	Elongation factor 1-β
	DDB0191102	Clathrin-adaptor medium chain apm 1
		hypothetical proteins
		proteins involved with the actin cytoskeleton:
	DDB0214810	Calcium-regulated actin bundling protein
	DDB0191108	S-adenosyl-L-homocysteine hydrolase
		metabolic proteins (e.g. fructose-bisphosphate aldolase)
DdCP∆N		
	DDB0191160	EB1
	DDB0220500	TACC
	DDB0215409	GTP-binding nuclear protein RAN/TC4
	DDB0216177	Dynamin like protein GTPase
		proteins involved with the actin cytoskeleton:
	DDB0219577	Filopodin (talin homologue)
	DDB0216426	Rubredoxin reductase
		hypothetical proteins

Table 4 DdEB1- and DdCP224-interacting proteins identified by TAP

Besides these contaminants, TAP analysis revealed many potential interactors of DdCP224 and DdEB1. The interactors shown in *Table 4* were selected from data resulting from several independent purifications and subsequent mass spectrometrical analysis (see materials and methods 4.11.1). The complete listing of all proteins identified can be seen in appendix (1).

With DdEB1 as a TAP-tagged bait, DdCP224 was detected as an interactor and vice versa *(Table 4*). The interaction between DdEB1 and DdCP224 was previously known and proven both by co-immunoprecipitation and co-localization (Hestermann & Gräf, 2004). The other interactors discovered were specific for DdEB1. The protein imidazoleglycerol-phosphate synthase detected in this study was also found to be associated with centrosomes in a project identifying centrosomal proteins in *Dictyostelium* (Reinders *et al.*, 2005). Other proteins identified as putative DdEB1 interactors were a guanine nucleotide-binding protein, heat shock protein Hsp70Bb and several actin-associated proteins. More difficult to distinguish as real interactors or contaminants are the metabolic proteins like glucose-6-phosphate dehydrogenase.

As stated above, DdEB1 was detected as an interactor with both the N- and the C-terminal part of DdCP224. Additionally, the GTP-binding nuclear protein RAN/TC4 seems to interact with both parts of DdCP224 as well and was further identified in a project looking for centrosomal proteins in *Dictyostelium* (Reinders *et al.*, 2005). Apart from that, interactors discovered with the N-terminal part of DdCP224 differ from the ones detected with the C-terminus. Among the putative interactors identified only with the N-terminal part of DdCP224 were tubulin, the elongation factors 1-β and 1-γ, the medium chain of Clathrin-adaptor apm 1 and several actin-associated proteins as well as metabolic proteins. Moreover, several unknown, hypothetical proteins were discovered.

Specifically for the C-terminal part of DdCP224 TACC (Transforming Acidic coiled Coil Containing) was identified along with a Dynamin like protein GTPase, some actin-associated proteins and hypothetical proteins.

The high number of bands present in all tandem affinity purifications *(Figure 21* and *Figure 20*) suggested the existence of further, heretofore unidentified interactors of DdCP224 and DdEB1. Not all these bands could be excised and subjected to mass spectrometry and an approach to entirely cut up each lane into fifty slices yielded only the most prominent proteins of each slice (data not shown). To assess the nature of the interactions found in this screen, an individual verification and characterization of the detected interactors is necessary.

As a first interactor to be subjected to further analysis DdTACC was selected, since its *Drosophila* homologue had been described as an interactor of the protein minispindles, representing the DdCP224 homologue in this organism (Lee *et al.*, 2001).

3 DdTACC1

The family of TACC (Transforming Acidic Coiled-coil Containing) proteins was originally found in mammals, where members of this family are overexpressed in cancer cell lines and transformed mouse cells (Still *et al.*, 1999). In *Drosophila* it was discovered in a microtubule spin down experiment (Gergely *et al.*, 2000b).
Here, DdTACC was identified by Tandem affinity purification in a band of about 200 kDa along with DdCP224, as can be derived from *Figure 21*.

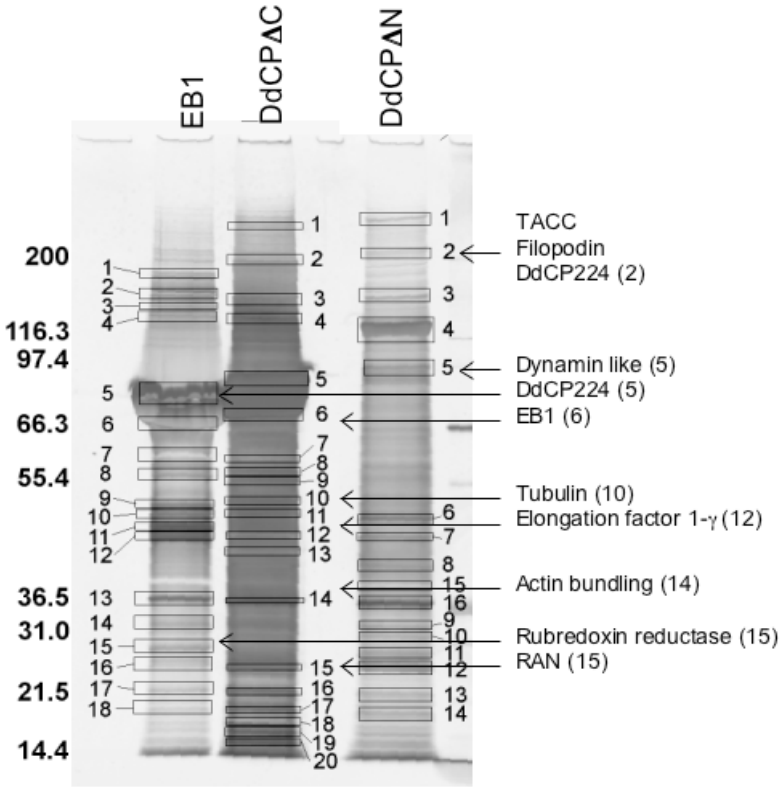

Figure 21 Example of a silver stained SDS gel showing final eluates and bands cut out for mass spectrometry. Arrows indicate position of bands; band numbers are shown in brackets.

This size is slightly larger than the predicted size of 175 kDa but not unusual for TACC proteins (Peset *et al.*, 2005).

3.1 Sequence Analysis

Sequence analysis of DdTACC at DictyBase (http://www.dictybase.org) was performed to identify the surrounding sequences and revealed an open reading frame of 4,529 bp, which contains one intron between bp 3,952 and 4,043 and corresponds to a protein of 1,478 aa (full sequences and flanking regions shown in Appendix).

According to a study performed by Iranfar et al. where probes were prepared from total RNA collected at 2 h intervals as well as from time-averaged reference RNA and superscript II DNA polymerase (Invitrogen, Carlsbad, USA) was used to incorporate either Cy5- or Cy3-conjugated dCTP (Amersham, St. Louis, USA) into DNA (Iranfar et al., 2003), DdTACC expression *(Figure 22*) is not developmentally regulated and quite stable throughout *Dictyostelium discoideum* lifecycle.

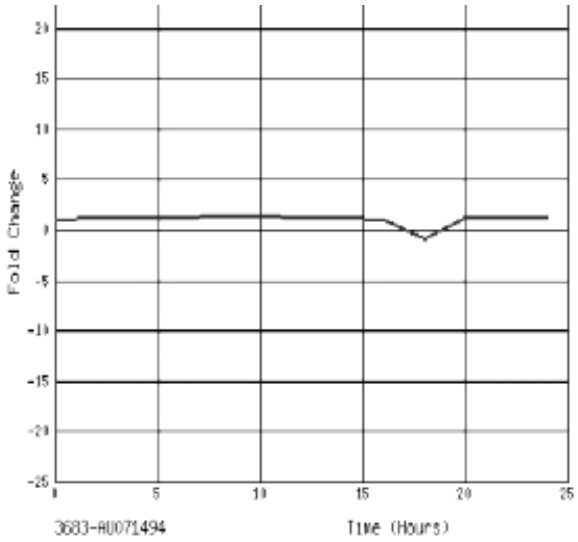

Figure 22 DdTACC expression profile. Expression data are averaged from 4 microarray experiments on Dictyostelium discoideum strain AX4 cells developed on filters. Samples were collected every 2 hours and analyzed as described (Iranfar et al. Eukaryot Cell. 2003 2:664-670). Values (y-axis) are the fold change of the ratio between the level of gene expression at a given time point and in a time averaged sample. All values were normalized to 1 at the time of initiation of development (T = 0) for ease of visualization. Figure taken from www.dictybase.org. Provided by Negin Iranfar, Danny Fuller, and William F. Loomis, Cell and Developmental Biology, UCSD, La Jolla, CA.

Like all members of this protein family, DdTACC displays only homologies of the C-terminal 200 aa in a blast search at http://www.ebi.ac.uk/blastall/index.html, which corresponds to the conserved so called TACC domain.

An alignment with TACC domains of different species as illustrated in *Figure 23* revealed that even within this homologous region, only between 22 % and 27 % sequence identity occurs. This rather weak homology is common for members of this family, as for example, the homologies between human and *Drosophila* members of the family are also only between 25 % and 30 %.

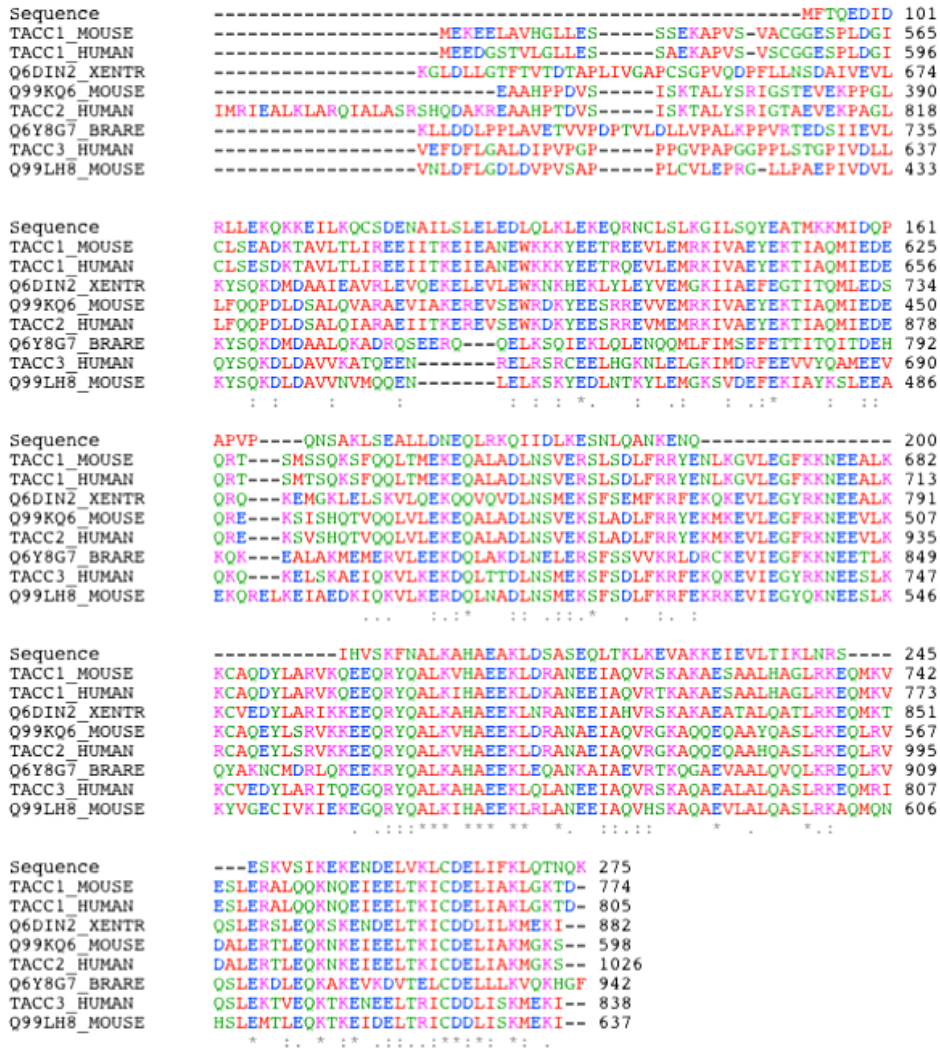

Figure 23 Homologies between DdTACC and other members of the TACC family. Pair wise identities: mouse 1: 27 %; human 1: 27 %*; Xenopus* 3: 24 %; mouse 2: 26 %; human 2: 26 %; *Danio rerio* 3: 24 %; human 3: 22 %; mouse 3: 25 %. Alignment was generated using CLUSTAL (1.0) multiple sequence alignment (http://www.ebi.ac.uk/clustalw/) (Thompson *et al.*, 2000). Sequences identities are marked with * in case of conservation throughout the whole set of sequences and with: and . in case of partial conservation. Pair wise scores are calculated as the number of identities in the best alignment divided by the number of residues compared (gap positions are excluded). Both of these scores are initially calculated as percent identity scores and are converted to distances by dividing by 100 and subtracting from 1.0 to give number of differences per site.

3.2 Creation of a GFP-TACC domain fusion protein

Since members of the family of TACC proteins only show homology within the TACC domain (Gergely *et al.*, 2000a; Gergely, 2002), this homologous part of *Dictyostelium* TACC was chosen for a GFP fusion construct (pKK14).

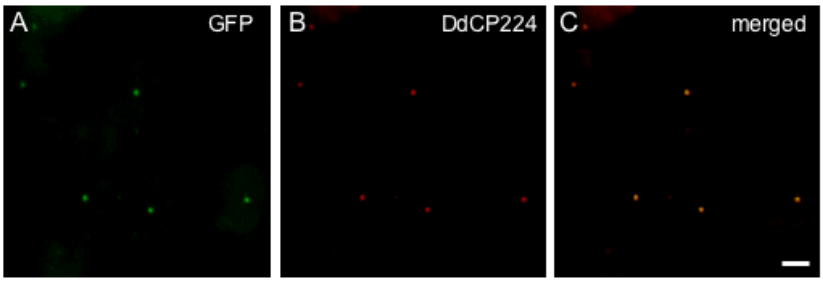

Figure 24 Localization of TACC domain. Cells expressing GFP-TACC (**A**) were fixed with methanol and stained with antiDdCP224mAb 2/165 (**B**). The merged image (**C**) shows the colocalization of both signals. Bar 2.5 µm.

The fusion protein clearly localized to the centrosome, as revealed by the additional staining with a centrosome-specific anti-DdCP224 antibody (*Figure 24*).

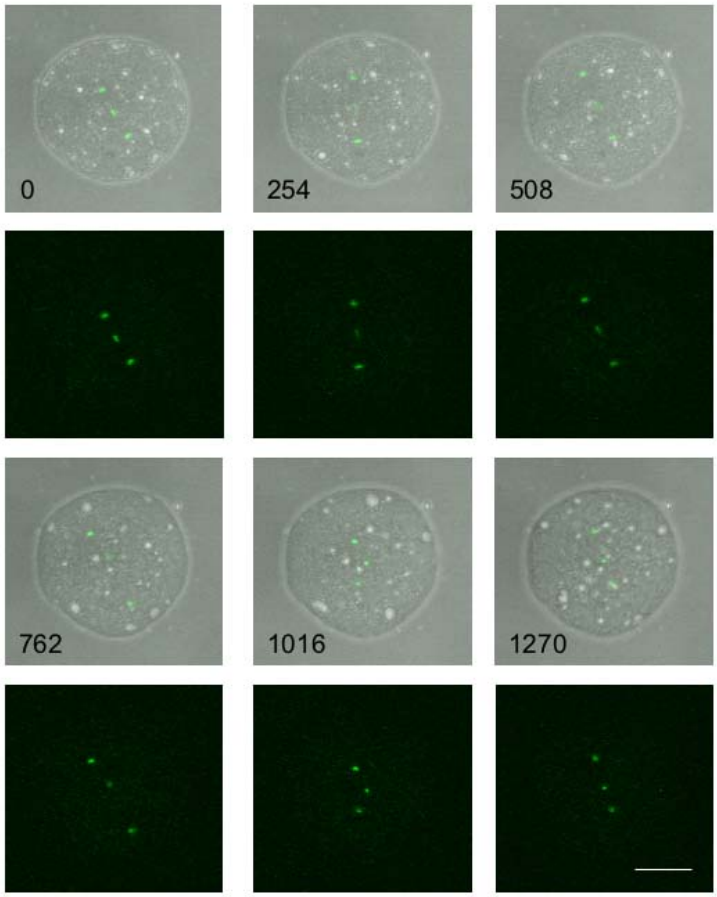

Figure 25 Localization of TACC domain during mitosis. Pictures were taken from a movie; time (s) is indicated in lower left corner of pictures. Upper rows show phase contrast of cells, lower rows matching GFP fluorescence pictures. GFP TACC stains both centrosomes as well as a structure in the kinetochore region throughout telophase. Bar 5 µm.

In contrast to other species where TACC is only detectable at the centrosome during mitosis (Barros *et al.*, 2005), it was present at the *Dictyostelium* centrosome in interphase cells as well as during telophase as displayed in *Figure 25*. The kinetochore region is stained in addition to both centrosomes.

3.3 Co-immunoprecipitation with DdCP224

To confirm the interaction between DdCP224 and DdTACC, a co-immunoprecipitation experiment was undertaken utilizing the available strain expressing GFP-TACCdomain. A protein extract of this strain (KK14) was fused to beads using either a GFP or a monoclonal anti-DdCP224 antibody (2/165). In both cases, a co-immunoprecipitation of GFP-TACCdomain and DdCP224 could be detected *(Figure 26*). This experiment not only confirmed the interaction of DdTACC and XMAP215 family proteins, but also revealed that the TACC domain was sufficient for an interaction with DdCP224.

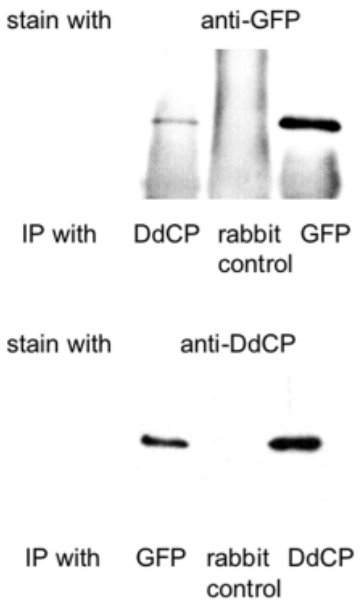

Figure 26 Co-immunoprecipitation with DdCP224. Co-immunoprecipitation of DdCP224 and GFP-TACC. For the upper panel, a cytosolic extract from a strain expressing GFP-TACC was used; the blot depicted in the lower panel was performed using an extract from wild type cells (AX2). For the upper blot, a monoclonal anti-DdCP224 antibody (2/165) was used for immunoprecipitation and an anti-GFP antibody (Faix *et al.*, 2001) was used for staining of the blot. The lower blot shows the inverted situation with the anti-GFP antibody used for immunoprecipitation and the DdCP224 antibody used for staining.

3.4 Purification of TACC domain by MBP fusion

To be able to express and purify sufficient amounts of protein to generate an antibody against DdTACC, the TACC domain was expressed as an MBP fusion protein (pKK20). The fusion protein was expressed and purified as described in materials and methods. Purification yielded pure MBP-TACC, which can be seen in *Figure 27*.

Like the full-length native TACC, the fusion protein is also detected at a larger than expected size which is in accordance to findings in other organisms (Peset *et al.*, 2005).

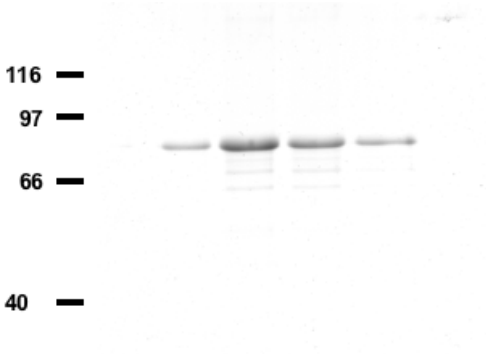

Figure 27 Expression of MBP-TACC. Several fractions of one purification were subjected to SDS gel electrophoresis and the resulting gel was stained with Coomassie. Bands visible at about 80 kDa correspond to MBP-TACC. Sizes of ladder are given in kDa.

3.5 Generation of polyclonal antibodies against TACC domain

To generate polyclonal antibodies against the DdTACC domain, the purified fusion protein as described above was send to *Pineda Antikörperservice* (Berlin). In advance, preimmunesera were tested by immune fluorescence microscopy, and only rabbits that did not yield much background fluorescence staining were used. All sera obtained from the immunizations were tested in Western blot analysis as well as immune fluorescence microscopy (*Figure 28*).

After more than 90 days of immunization, sera of both animals displayed sufficient results. In order to get a clearer signal in Western blot analyses, sera were purified as described in material and methods 4.10. Since this did not significantly improve the signal, additionally stained bands in Western blots are most likely due to degradation of the protein or cross-reactions of the DdTACC antibodies. In immune fluorescence microscopy, a clear colocalization with the GFP signal could be shown (*Figure 28*).

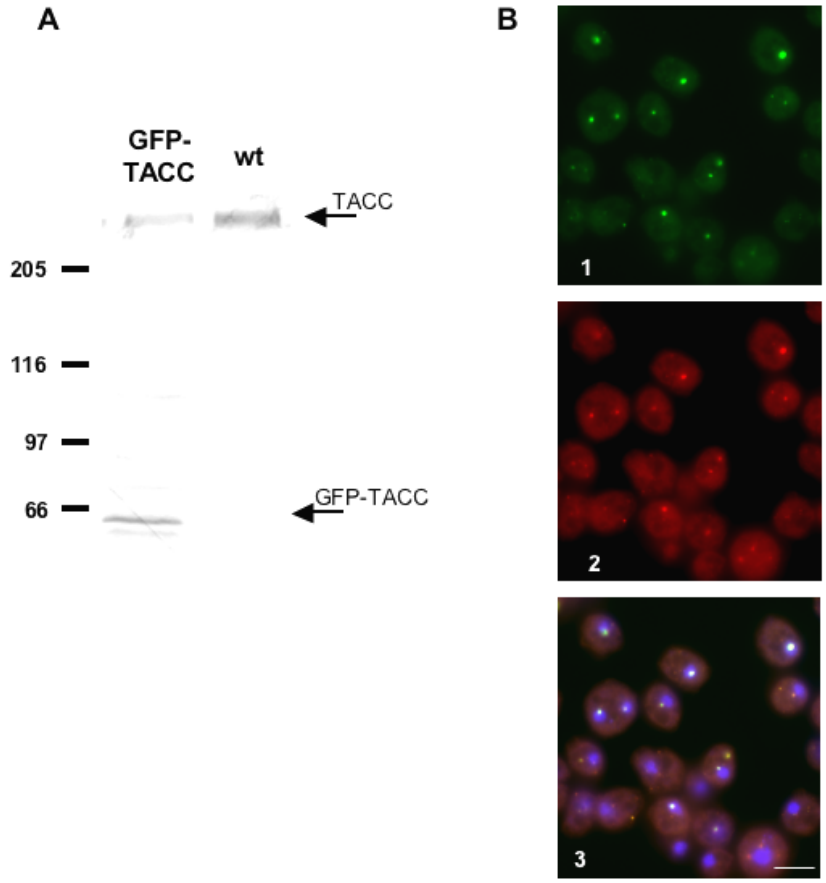

Figure 28 DdTACC-antibody in Western blot analysis and immune fluorescence microscopy. A Expression of DdTACC and GFP-TACCdomain detected with a DdTACC-antibody. Native DdTACC can be found at about 200 kDa, the TACC domain with addition of GFP could be detected at about 60 kDa. All sizes are given in kDa. **B** Cells expressing GFP-TACC (1) were fixed with methanol and stained with antiDdTACC (2). The merged image (3) shows the colocalization of both signals and nuclei stained with DAPI (shown in blue). Bar 10 µm.

3.6 Isolation of centrosomes

Even though all GFP-TACCdomain expressing strains as well as stainings with anti TACC antibodies resulted in a localization at the centrosome that was confirmed by an additional staining with DdCP224, a further experiment was conducted to verify the nature of DdTACC as a centrosomal component.

In a centrosome purification (Schulz *et al.*, 2006) isolated centrosomes devoid of microtubules are obtained (see materials and methods). These were subjected to immune fluorescence microscopy and Western blot analysis where TACC signals could still be detected (*Figure 29*). As proteins that can be found at the centrosomes independent of microtubules are generally considered to be bone-fide centrosomal proteins (Rehberg & Gräf, 2002), this experiment verified the nature of DdTACC as a true centrosomal protein.

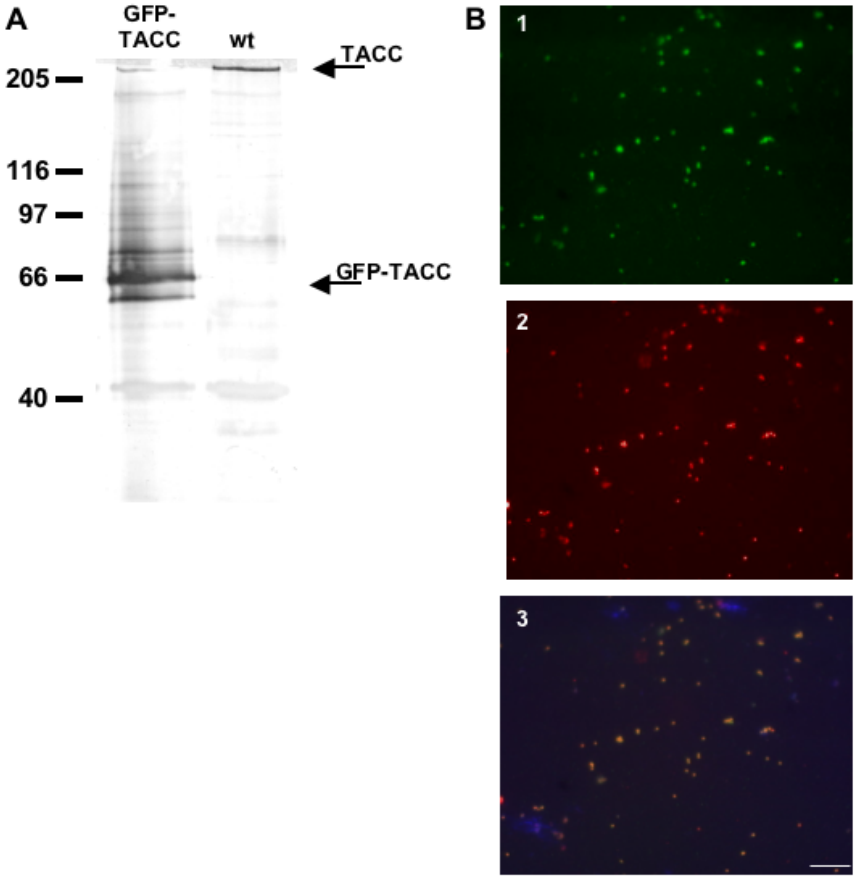

Figure 29 DdTACC is a centrosomal component. A Detection of DdTACC and GFP-TACCdomain detected with a DdTACC-antibody in a preparation of isolated centrosomes. Native DdTACC can be found at about 200 kDa, the TACC domain with addition of GFP could be detected at about 60 kDa. All sizes are given in kDa. **B** Purified centrosomes from a strain expressing GFP-TACC were fixed with methanol and stained with antiDdCP224 (2/165) (1) GFP fluorescence. (2) Anti-DdCP224 signal. (3) merged image shows the colocalization of both signals. Bar 5 µm.

3.7 Deletion construct

To define the function of DdTACC an attempt to generate mutants that lack the protein was performed. For disruption of the Ddtacc sequence, an 800 bp fragment of the 5' utr and the 800 bp fragment containing the TACC domain were cloned into a vector containing a blasticidin-resistance-cassette (bsr) (pIS102) (*Figure 30*). The plasmid pKK19 contained these two sequences surrounding the bsr cassette (*Figure 30*), so that by homologous integration more than 3,500 bp of DdTACC coding sequence would be substituted by the bsr cassette. *Dictyostelium* cells were transformed with the linearised targeting vector.

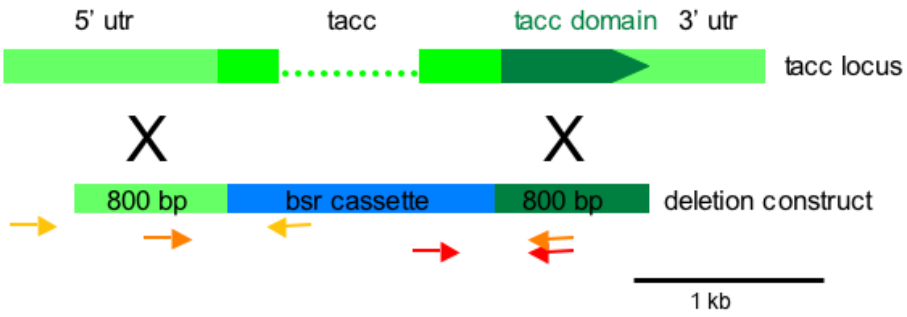

Figure 30 Disruption of the DdTacc gene. The DdTACC knock out was attempted using a targeting vector consisting of two genomic sequences fused together sparing out more than 3,500 bp of tacc encoding sequence. The blasticidin selection cassette (bsr) was inserted in exchange. Due to its length, display of tacc coding sequence has been shortened (indicated by dots). Primers used for the PCR screenings are shown in a specific color code for each pair.

More than five independent transformations yielded only very few surviving transformants. These were tested by several PCR screenings with primers within and outside of the construct to identify KO candidates. Unfortunately, all clones that contained the disruption construct also still contained the wild type sequence detectable by PCR and also visible in Western Blot analyses and immune fluorescence (data not shown).

Therefore, no DdTACC knockout strain could be generated in this study suggesting that the gene product is essential.

IV Discussion

1 Yeast two hybrid screening

As a first approach to look for interactors of DdCP224 and DdEB1, two MAPs, which are associated with both microtubule plus ends and the centrosome in *Dictyostelium discoideum* (Gräf *et al.*, 2000; Gräf *et al.*, 2003; Rehberg & Gräf, 2002), a yeast two hybrid screen was performed. Since the C-terminal 460 amino acids of DdCP224 are sufficient for centrosomal binding (Hestermann *et al.*, 2002), whereas a construct consisting of the N-terminal 813 amino acids localizes to the cell cortex (Hestermann & Gräf, 2004) and the full length protein would be too large to use, the screen for interactors was performed separately for these two parts. For DdEB1, a full-length construct was used.

1.1 Five putative interactors identified

Upon the numerous interactors identified when growing the clones on medium stringency LWH^- medium (887 (350 DdCP c-term, 237 DdCP n-term, 300 DdEB1)) only 132 (26 DdCP c-term, 56 DdCP n-term, 50 DdEB1) were still able to grow under more stringent conditions on $LWHA^-$ media. Performing a β Galactosidase assay, even less clones held up to the selection so that only 91 (3 DdCP c-term, 48 DdCP n-term, 40 DdEB1) positives could be subjected to further analysis. After determination of the length of the inserts in these hereby identified library plasmids, it turned out that none were larger than 1,000 bp, most being smaller than 100 bp. This leads to the conclusion that the library may only have contained small fragments. This could be due to a premature end of both the random and the oligo (dT) priming to generate this library, which is most likely caused by poor RNA quality. Unfortunately, all library plasmids identified as putative interactors contained only very small fragments of which most contained stop codons in the first part of all reading frames or only polyA stretches. Also a lot of ribosomal proteins have been identified which are common false positives in yeast two hybrid screens according to a study by Erica Golemis (http://www.fccc.edu/research/labs/golemis/main_false.html). So, at the end of the selection process five putative interactors could be used for further analysis.

1.2 Putative DdEB1 interactors show no co-localization

For a first assessment of the putative interactors identified in this screen, they were expressed as N-terminal GFP-fusion proteins. None of these GFP fusion proteins localizes to the centrosome or the microtubule tips or along microtubules.

EB1-6 showed a clear localization to the Golgi apparatus, which is at least associated with the centrosome and for an over expression mutant of the DdCP224 N-terminus, Golgi dispersal has been observed (Hestermann & Gräf, 2004) but this is insufficient to explain a physiologically relevant interaction with DdEB1. Although, Golgi dispersal has been described as a phenotype for a number of proteins that also interact with Dynein (e.g. Hook (Malone *et al.*, 2003)), which co-immunoprecipitates with DdEB1 (Hestermann & Gräf, 2004). Recently, a protein (centrosomin's beautiful sister) has been described to link the Golgi apparatus to the centrosome during the cell cycle (Eisman *et al.*, 2006).

Calcineurin B has been characterized in *Dictyostelium discoideum* as a subunit of the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin and is unconventionally spliced into two isoforms (Aichem & Mutzel, 2001). No specific cellular localization of this protein could be found and apart from a developmental regulation and its unusual splicing, Aichem et al (2001) were not able to clearly determine its physiological function. In other organisms it has been described to be involved in T-cell activation (Crabtree & Clipstone, 1994) and regulation of cell cycle arrest in yeast (Cyert & Thorner, 1992). None of these previously described functions point to a specific interaction with EB1. Since also all attempts to co-immunoprecipitate it with DdEB1 or get positive results from a GST-pulldown failed, it is considered to be a false positive as well.

For the putative interactor EB1-67, no homologous proteins could be found and it has not been characterized in *Dictyostelium discoideum* yet. Since its interaction also was not provable by other means, it most likely represents another false positive.

In general, colocalization is considered to be a good indicator for a physiologically relevant interaction between proteins. Nevertheless, an interaction between these putative interactors and DdEB1 is still possible without a strong colocalization. Unfortunately, the putative interactors did not only lack a clear colocalization with DdEB1, but it was also not possible to prove the interaction by other means.

Taken together, it is considered unlikely that they do really interact with DdEB1 in *Dictyostelium discoideum* in a physiologically relevant way. Therefore the analysis of all three detected putative DdEB1 interactors was discontinued.

1.3 Putative interactors of N-terminal part of DdCP224 reveal autophagy protein

The two putative interactors of the N-terminal part of DdCP224 displayed no colocalization with DdCP224. DdCPn-term-27 localizes also to the perinuclear region which could imply a proximity to the centrosome, that is attached to the nuclear envelope (Okuda *et al.*, 2000) and therefore might interact with DdCP224 at the centrosome. The N-terminal part of DdCP224 alone does not show a centrosomal localization (Hestermann & Gräf, 2004), thus a real interaction between this protein, of which no homologous proteins have been found, and the N-terminal part of DdCP224 is at best doubtful. Given that no other means could prove the interaction, the investigation of this interactor was not persuaded any further.
Also for the other detected putative interactor of this part of DdCP224, Atg 8, the subcellluar localization does not correspond with DdCP224 localization, but marks the autophagosomes and has been assigned a function in autophagy (Otto *et al.*, 2003; Otto *et al.*, 2004). From this function no direct interaction with DdCP224 can be determined, but strikingly, it is homologous to other known MAPs. It can be speculated that Atg 8 plays a role in the transport of autophagosomes along microtubules. For example, it has been shown for the Arabidopsis homologue to Atg 8 to be involved in autophagy and to cosediment with microtubules (Ketelaar *et al.*, 2004), so a similar situation may be present in *Dictyostelium discoideum* as well, but would need further investigation. This project was abandoned, as none of the other methods used confirmed the interaction with DdCP224.

1.4 Conclusions on yeast two hybrid screening

In addition to finding many common false positives, the putative interactions selected for further analysis could not be proven using another method (colocalization, coprecipitation and GST-pulldown). Even according to the inventors of this method, multiple reasons for the detection of false positives are known (Bartel, 1993). However, since the yeast two-hybrid system is able to detect even very transient

interactions (Gavin, 2002), it cannot be ruled out that the interactors found are too transient to be detected by another method.

The fact that none of the known interactors of DdEB1 and DdCP224 could be found by this approach points to a general deficiency in the library used. No publishable results have been produced by other researchers using this library, including the original producer (J. Faix, A. Müller-Taubenberger, personal communication). Although it is possible that hybrids of known interactors did form but were unable to enter the yeast nucleus, which is necessary for the expression of the reporter genes.

One has to keep in mind that many factors contribute to the success of a yeast two hybrid screen, according to the manufacturers it is depended upon the yeast strains used, the specificity and size of the target protein, the library vector and the source of the cDNA (Clontech manual). Nevertheless, a serious problem with the strain used or the bait proteins should result in no putative interactors to be detected, which was not the case in this study, where more than 800 clones were initially found. Therefore it is very likely that the lack of identified new and known interactors of DdEB1 and DdCP224 is due to the poor quality of the library used.

2 A novel method to search for protein interactions in *Dictyostelium discoideum*: Tandem affinity purification

Due to the fact that none of the putative DdEB1 and DdCP224 interactors detected by the yeast two hybrid approach could be verified by other means, the search for interactors had to be pursued utilizing another method. The promising results of the Tandem affinity purification method originally developed in yeast (Puig *et al.*, 2001; Rigaut *et al.*, 1999) made it a good candidate and it was therefore decided to adapt it to *Dictyostelium discoideum.*

TAP is a chromatographic method that had several successful applications in describing novel protein interactions in *S. cerevisiae* (Gavin *et al.*, 2002; Gingras *et al.*, 2005; Shevchenko *et al.*, 2002; Song *et al.*, 2002), and in the closely related fission yeast (Gould *et al.*, 2004; Horn *et al.*, 2005; Tasto *et al.*, 2001). For mammalian cell lines several approaches of modified Tandem affinity purification have been reported recently (Drakas *et al.*, 2005; Li *et al.*, 2004) and the method was also successfully used in *C. elegans* and *Drosophila* (Rubio *et al.*, 2005; Veraksa *et al.*, 2005) .

In comparison to the yeast two hybrid system, a great advantage of Tandem affinity purification in *Dictyostelium discoideum* is that protein interactions can be assessed in the organism of interest itself. Therefore, all problems resulting from the expression of the proteins in yeast and the fact that interacting partners have to be able to get to the nucleus for the interaction to be detected are eliminated. Also, since no cDNA has to be generated, problems during the creation of the library are eliminated as well. Additionally, weakly expressed binding partners of DdEB1 and DdCP224 are more likely identified by a sensitive method such as mass spectrometry than by yeast two-hybrid screening of a cDNA library, since cDNAs of weakly expressed proteins are usually underrepresented in these libraries and hence, difficult to identify. Further, previous studies have proven that interactions undetectable by yeast two hybrid analysis could be identified using TAP followed by mass spectrometry (Gavin *et al.*, 2002; Rigaut *et al.*, 1999). Moreover, the number of false positive interactors found in previous studies was very low (Puig *et al.*, 2001). Other great advantages of TAP are the gentle physiological buffers and elution conditions that can be used throughout the whole purification process and prevent disruption of protein-protein interactions within the protein complexes of interest. This is enabled through the elution from the first column via TEV protease (Senger *et al.*, 1998), which prevents elution of unspecifically bound proteins lacking the TEV-protease recognition site. The recent completion of the *Dictyostelium* genome project (Eichinger *et al.*, 2005) opened up a good perspective for a proteomics approach to identify putative interactors. Thus, although transient interactions may be missed by the TAP method and are sometimes only detected by yeast two-hybrid (Gavin *et al.*, 2002), the adaptation of the TAP method to the *Dictyostelium* model system was a promising approach to identify new and reliable interactors of DdEB1 and DdCP224 both at the centrosome and at microtubule plus ends.

2.1 All constructs are expressed and considered fully functional

To allow an optimal screening for interactors, the conditions should be as close to the native ones as possible. Thus, expression levels should best be kept close to the levels of the wild type protein. Since both DdEB1 and DdCP224 are present during the whole cell cycle in sufficient amounts, expression of the tagged fusion proteins under the control of the constitutively active actin 15 promotor was suitable and resulted in expression levels comparable to the native proteins *(Figure 17*). The

greatest yield for Tandem affinity purification is expected upon exclusion of the native protein and substitution by the tagged version. In case of DdCP224, a knockout is considered to be lethal (Gräf *et al.*, 2003), therefore all constructs were expressed in presence of the wild type protein. Because the complexes DdEB1 and DdCP224 are part of are expected to contain multiple copies of these proteins, the presence of wild type proteins during the purification should not cause any problems, since one tagged member per complex is sufficient for purification. Although, in case of DdCP224, which was split in a C- and an N-terminal part, it cannot be ruled out that the detected interactors might be purified due to a possible interaction with the native full length protein also present in the complex.

Another important prerequisite for Tandem affinity purification is the accessibility of the recognition sequence for the TEV protease. This was tested by subjecting protein extracts from strains expressing the fusion proteins to TEV protease cleavage (*Figure 17*). Taken together, the fusion of the protein of interest with the TAP-tag did not interfere with the accessibility of the TEV protease cleavage site. This was true for all three constructs used in this study but should be tested for all new constructs using the TAP-tag. The test is also a good indicator for optimal cleavage conditions and the quality of the TEV protease.

Full functionality of the fusion proteins is most important for a successful screen for physiologically relevant interactors. In this study, functionality was assumed from the expected localization of all three fusion proteins, since no easily detectable knockout phenotype is present in case of DdEB1 and for DdCP224 a knockout is assumed to be lethal (Gräf *et al.*, 2003). Additionally, the detection of known interactions (see below) was a good indicator for the functionality of the fusion proteins.

2.2 Identified proteins are bait specific

SDS-gel electrophoresis revealed that Tandem affinity purification resulted in a strong enrichment of the tagged proteins from cytosolic extracts *(Figure 20*). This is a good indication that the conditions used were suitable for the binding of the tag to the affinity columns as well as for the selective elution of the tagged complexes. Subjection of individual protein bands to mass spectrometrical analysis (see materials and methods 4.11.1) showed that the faint bands visible in controls of mock purifications of the wild type strain AX2 and strain KK4 (data not shown), which

contains the TAP-tag without a bait attached to it, represented mainly ribosomal proteins as well as discoidin and actin.

Given that ribosomal proteins were found in both controls as well as with all three strains expressing the TAP-tagged bait proteins, they can be considered as unspecific contaminants. Actin is very abundant in *Dictyostelium* cell extracts, therefore its appearance may simply be due to remnants of protein extract in the final eluate. Discoidin has first been described as a lectin (Poole *et al.*, 1981). This property may cause a significant affinity to conjugated carbohydrate-beads as they are used during the purification steps and may explain its detection even in purifications from wild type cells expressing no TAP-tag at all. Ribosomal proteins as well as very abundant proteins such as actin are generally frequent contaminants in TAP (Rigaut *et al.*, 1999; Shevchenko *et al.*, 2002). Another frequent contaminant in TAP experiments is calmodulin, which was detected with the TAP-tag by itself (KK4) as well as with all three TAP-tagged constructs as bait. This may result from an interaction of calmodulin with the calmodulin binding peptide of the TAP-tag. Yet, in case of the TAP-tagged microtubule associated proteins used in this study, it could also reflect a real affinity of calmodulin to complexes containing these proteins, since a natural interaction of calmodulin with microtubules has been described previously (Moore *et al.*, 1998; Ueno *et al.*, 2003). Besides these contaminants, TAP analysis revealed many potential interactors of DdCP224 and DdEB1.

2.3 DdEB1 interactors

With DdEB1 as a TAP-tagged bait, DdCP224 was detected as an interactor and vice versa (*Table 4*). This interaction was previously known and proven both by co-immunoprecipitation and co-localization (Hestermann & Gräf, 2004) and further illustrates the ability of the method to yield reliable interactors. Another protein identified with tagged DdEB1 is imidazoleglycerol-phosphate synthase, which plays a role in histidine biosynthesis in pro- and eukaryotes (Alifano *et al.*, 1996). It was also found to be associated with centrosomes in a project identifying centrosomal proteins in *Dictyostelium* (Reinders *et al.*, 2005) and therefore seems to be a good candidate for a genuine centrosomal protein, which might very well be a physiologically relevant interactor of DdEB1. A guanine nucleotide-binding protein found associated with DdEB1 might be an example for a link of EB1-proteins to RhoGTPase signalling pathways. For example, in *Drosophila* Concertina, a RhoGTPase, regulates the

interaction of EB1 and RhoGEF, which uses this interaction to travel to the cell cortex on the tips of growing microtubules (Rogers *et al.*, 2004). A further, potentially specific, interactor of DdEB1 was heat shock protein Hsp70Bb. Recently, such an association between an EB1-family protein family and HSP70 was described, however, it is mediated by a third protein (Wadle *et al.*, 2005). Since the components of protein complexes identified by TAP analysis do not necessarily have to interact directly with each other, such an indirect interaction could also be assumed for *Dictyostelium*. Several potential interactors of DdEB1 and DdCP224 (see below) are actin-associated proteins. This is not surprising, because microtubule plus end-binding proteins such as DdCP224 and DdEB1 have turned out not only to regulate microtubule dynamics but also to mediate interactions of microtubules with the actin cytoskeleton (Hestermann *et al.*, 2002). The DdEB1-binding protein HSP70, for example, also plays a role in actin polymerization (Haus *et al.*, 1993). Likewise, the 34 kDa subunit of the Arp 2/3 complex might as well be involved in linkage of the microtubule cytoskeleton to cortical actin (Bretschneider *et al.*, 2004; Cvrckova *et al.*, 2004). Another potential DdEB1 interactor involved with the actin cytoskeleton is rubredoxin reductase. It contains two ADF domains (actin depolymerization factor/cofilin-like domains) (Cvrckova et al., 2004) and is very similar to human and mouse twinfilin, a tyrosine kinase and actin monomer binding protein.

2.4 DdCP224 interactors

As mentioned above, the interaction between DdCP224 and DdEB1 was also confirmed when DdCP224 was used as a bait for TAP analysis and DdEB1 was identified as an interactor both with the N-terminal and C-terminal part of DdCP224. Consequently, it appears likely that at least one of these interactions is indirect and mediated by other proteins. This view is further supported by the fact that we were unable to detect any direct interaction between DdCP224 and DdEB1 in a yeast two-hybrid assay (data not shown). The small GTPase Ran that was also found with both parts of DdCP224 was detected as well in a project identifying the centrosomal proteome in *Dictyostelium (Reinders et al., 2005)*. Ran together with DdCP224 may be involved in microtubule organization at the centrosome, since in *Xenopus* extracts both Ran and the DdCP224 homologue XMAP215 have been shown to be essential for self-organization of microtubule asters together with the γ-tubulin ring complex and dynein (Ohba *et al.*, 1999; Wilde & Zheng, 1999). Ran and DdCP224 may also

cooperate in centrosome duplication, a process in which both proteins seem to be involved (Caudron *et al.*, 2005; Gräf *et al.*, 2000; Gräf *et al.*, 2003; Wang *et al.*, 2005). Apart from that, interactors found with the N-terminal part of DdCP224 differ from the ones found with the C-terminus. Tubulin was found with the N-terminal part of DdCP224, which is in accordance to the findings with its human homologue ch-TOG, where some 600 aa of the N-terminus are sufficient for binding of tubulin dimers (Spittle *et al.*, 2000). In previous studies we showed that the N-terminal part of DdCP224 is necessary for microtubule binding since the C-terminal part alone localizes to the centrosome only (Hestermann *et al.*, 2002). On the contrary, in case of XMAP215, the *Xenopus* homologue, it is known that the C-terminal part also interacts with microtubules and only the microtubule-stabilizing activity is restricted to the N-terminus (Popov *et al.*, 2001).

Several other potential DdCP224 interactors have been identified here for the first time and may pinpoint still unknown functions of XMAP215-family proteins. All these proteins have in common that they are associated with the cytoskeleton and thus they must be considered as real direct or indirect interactors of DdCP224. Elongation factors, for example, have been known to interact with the cytoskeleton (Moore & Cyr, 2000; Ueno *et al.*, 2003), therefore the detection of EF1-γ and β is quite plausible. Clathrin, for instance, plays a role at the mitotic spindle and the heavy chain binds directly to spindle microtubules (Royle *et al.*, 2005; Schulz, 2006) and was further found in a comparative proteomics approach to identify novel centrosomal components in *Dictyostelium discoideum* (Reinders, 2005) and proven to be a genuine centrosomal protein (Schulz, 2006). Therefore, it is not unlikely that also the medium chain of the clathrin adaptor is involved with the cytoskeleton, even though only a role in endosome organization has been described so far (Lefkir *et al.*, 2004). The G protein-binding protein found in addition to Ran may be another member of the regulatory machinery associated with GTP.

As in case of DdEB1, we also identified several actin-associated proteins such as the calcium regulated actin bundling protein (Furukawa *et al.*, 2003) when the N-terminal part of DdCP224 was used as a bait for TAP analysis. Another example is S-adenosyl-L-homocysteine hydrolase, a component of actin rods found in germinating spores, that has been localized at the cell cortex and the nucleus as it has been described for DdCP224 (Hestermann & Gräf, 2004; Kishi *et al.*, 2001; Yamada & Sameshima, 2004). The interactions with the N-terminus of DdCP224 are likely to be

mediated through the TOG domains, which contain several HEAT repeats. These HEAT repeats represent a protein-binding motif involved in various protein-protein interactions (Neuwald & Hirano, 2000).

Specific interactors of only the C-terminal part of DdCP224 were the TACC protein, which is described in detail below, and a dynamin-like GTPase. Dynamin was first discovered because of its binding to microtubules (Shpetner & Vallee, 1989) but in *Dictyostelium* its role in endocytosis has been more closely investigated, although its depletion also leads to multinucleated cells due to defects in cytokinesis (Wienke *et al.*, 1999). The talin homologue filopodin, a protein that nucleates actin assembly and provides a link between actin microfilaments and the plasma membrane (Kreitmeier *et al.*, 1995), is another example of an actin-associated protein identified in this screen. The actin-associated rubredoxin reductase, which was already identified as an interactor of DdEB1 (see above), was again found as an interactor of DdCP224 C-terminus. This may confirm a potential role as a member of the cytosolic DdCP224/DdEB1-complex and argues against co-purification as a contaminant together with actin itself.

Although actin itself was clearly present as a contaminant in our TAP eluates, the identified actin-associated proteins do not necessarily need to be contaminants as well, especially those, which were not identified in mock purifications. As already mentioned above, these proteins may represent true interactors of DdCP224 and DdEB1 which could be involved in the connection of microtubules and the actin cytoskeleton at the cell cortex (Hestermann *et al.*, 2002). Thus, any putative interaction of members of this group should be critically analyzed in further studies. Another group that is difficult to assess are the metabolic proteins found in this study. Though they are often ruled out as contaminants, several studies show an involvement of some metabolic enzymes with the cytoskeleton (Lloyd & Hardin, 1999). Most putative interactors found in this study appear to be reliable candidates for physiologically relevant interactions. For example the interaction between DdEB1 and DdCP224 that was detected with all three constructs is well proven by our lab (Hestermann & Gräf, 2004) and therefore nicely demonstrates the ability of the method to detect interactions. Another hint that the identified interactors are good candidates rather than false positives is the fact that, apart from a few overlapping findings, which are due to the presence of all baits within the same complex, the subset of interactors found is specific to each of the baits used. One example is that

the centrosomally localized DdTACC was found with the C-terminal part of DdCP224, which is sufficient for centrosomal localization of DdCP224 (Hestermann *et al.*, 2002). As another example, tubulin was found with the N-terminal part of the protein, which is necessary for the microtubule interaction of DdCP224. The low extent of overlap of the results of the individual screens with the three different bait constructs suggests that there is not only a single, large DdEB1/DdCP224 complex but that both proteins are also able to form individual cytosolic complexes in the absence of the other binding partner. One should keep in mind that native full length DdCP224 was still present in the cells used for the extraction of the TAP tagged complexes and might mediate indirect interactions in complexes with more than one DdCP224 protein present.

The high number of bands present in all tandem affinity purifications (*Figure 20* and *Figure 21)* suggested the existence of further, heretofore unidentified interactors of DdCP224 and DdEB1. Taken together, DdCP224 and DdEB1 seem to be not only members of a common cytosolic complex but are also able to form individual complexes with several other proteins as this would explain the finding of independent sets of interactors. Some further unidentified interactions might be transient or only present during development and, as a result, may not have been identified in this screen where growing vegetative cultures in axenic medium were used. To assess the nature of the interactions found in this screen, an individual verification and characterization of the detected interactors should be conducted.

2.5 DdTACC colocalizes and coprecipitates with DdCP224

As a first candidate, the interaction between DdTACC and the C-terminal part of DdCP224 was subject to a closer investigation. DdTACC was detected in a band of around 200 kDa *(Figure 21*), which is slightly larger than the predicted size of 175 kDa but not unusual for TACC proteins (Peset *et al.*, 2005). Since members of the family of TACC proteins only show homology in a C-terminal region of about 200 amino acids (Gergely *et al.*, 2000a; Gergely, 2002), this homologous part of *Dictyostelium* TACC was chosen for a GFP fusion construct (pKK14). It clearly localized to the centrosome, as revealed by the additional staining with a centrosome-specific anti-DdCP224 antibody (*Figure 24*). By contrast to other species where TACC is only detectable at the centrosome during mitosis (Barros *et al.*, 2005), it is present at the *Dictyostelium* centrosome during mitosis as well as during

interphase (*Figure 25* and *Figure 29*). Additionally, co-immunoprecipitation could be shown with both DdCP224 and GFP-TACC domain (*Figure 26*). Consequently, the interaction between DdCP224 and TACC that is known from several other species (Cullen & Ohkura, 2001; Lee *et al.*, 2001) could be verified in *Dictyostelium discoideum*.

2.6 Specific antibodies confirm localization detected with GFP-TACC domain

The polyclonal antibodies generated against the TACC domain of DdTACC showed the same staining pattern as the GFP signal of the GFP-TACC domain in repeated immunofluorescence experiments (*Figure 24* and *Figure 28*). In addition, they detected the GFP-TACC domain fusion protein in Western blot analysis as well as a band that corresponds to the size expected from the original TAP gel, where DdTACC was found in a band of more than 200 kDa. This is in accordance to findings in other species (Peset *et al.*, 2005) were TACC was also detected in bands of a larger size than the predicted one.
Therefore, the antibodies generated against DdTACC in this study are clearly able to specifically recognize DdTACC both in Western blot analysis and immune fluorescence microscopy.

2.7 DdTACC is a genuine centrosomal protein

Both the GFP signal and the antibody staining could be detected at isolated centrosomes and marked the corresponding bands in Western blot analysis. This confirms the localization of DdTACC at the centrosome, first discovered by colocalization with DdCP224. Since the purified centrosomes are devoid of microtubules (Gräf *et al.*, 1998), a localization in the vicinity of the centrosomes merely mediated by microtubules can be excluded for DdTACC and it is thus classified as a genuine centrosomal protein.

2.8 DdTACC expression seems to be vital to cells

In order to generate a *Dictyostelium discoideum* strain devoid of DdTACC several attempts were undertaken but all remained unsuccessful. Even though only one knockout construct *(Figure 30*) was used, a deficiency in the construct is unlikely, since the length (800 bp) and properties of the flanking regions are generally

considered to be suitable for homologous recombination in *Dictyostelium discoideum* (Kimmel & Faix, 2006). This corresponds to the finding that, in all species examined thus far, perturbations of TACC proteins lead to disruptions of cell cycle progression and/or embryonic lethality (O'Brien *et al.*, 2005), even if only one member of several ones present in the organism is deleted (Piekorz *et al.*, 2002). Therefore it is most likely that deletion of the only TACC member present in *Dictyostelium discoideum* affects cell cycle progression in such a drastic way that a knockout would be lethal.

3 Conclusions and outlook

Even though the yeast two hybrid system is a valuable tool to investigate protein-protein interactions, in the day and age of proteomics becoming available for more and more organisms, tandem affinity purification together with mass spectrometry provides an interesting approach to overcome downsides of the yeast two hybrid system. In this study, Tandem affinity purification was adapted for the use in *Dictyostelium discoideum* for the first time. The presence of TACC as a known interactor of XMAP215 proteins in the TAP tagged complex of DdCP224 demonstrates the credibility of this method. Together with the finding of DdEB1 with DdCP224 baits and vice versa, it also illustrates the fact that the conditions were mild enough to keep the complex intact throughout the various purification steps. TAP in *Dictyostelium discoideum* is an easy to use, reliable tool to shed light on complexes present in the cells and can be used to investigate many more complexes.

In addition, this study examined the interaction between DdCP224 and DdTACC. DdCP224 and DdTACC clearly do interact. The localization of DdTACC throughout the cell cycle could be observed and it is proven to be a genuine centrosomal protein. Despite these findings, the role of the only member of the TACC family of proteins present in *Dictyostelium discoideum* remains difficult to assess. Since the generation of a knockout of DdTACC was not feasible, its role during the cell cycle seems to be a vital one. This could be investigated in further studies using RNAi to down regulate the expression of DdTACC in a more differentiated way or by generating DdTACC underexpression mutants.

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VI Appendix

1 Proteins identified by tandem affinity purification

Bait	Protein	Mass in Da	DictyBase ID
DdCP224Cterminus	Actin	45126	DDB0220456
DdCP224Cterminus	Ankyrin	157057	DDB0186162
DdCP224Cterminus	Calmodulin	17151	DDB0214955
DdCP224Cterminus	Chr unknown00	395515	DDB0201696
DdCP224Cterminus	Chr unknown50 TACC1	173906	DDB0220500
DdCP224Cterminus	Class VII unconventional myosin	265548	DDB0185049
DdCP224Cterminus	Developmental protein	171954	DDB0220018
DdCP224Cterminus	Discoidin II	28575	DDB0215382
DdCP224Cterminus	Dynamin like protein	96277	DDB0216177
DdCP224Cterminus	Elongation factor 1-alpha	49662	DDB0191135
DdCP224Cterminus	Gamma-glutamyltranspeptidase	33875	DDB0203420
DdCP224Cterminus	GTP-binding nuclear protein RAN/TC4	24218	DDB0215409
DdCP224Cterminus	Guanylyl transferase	32462	DDB0217857
DdCP224Cterminus	Hypothetical 74.8 kDa protein	34911	DDB0190639
DdCP224Cterminus	Large subunit terminase	28715	DDB0217373
DdCP224Cterminus	Microtubule-associated protein CP224	223812	DDB0189914
DdCP224Cterminus	Microtubule-associated protein EB1	51789	DDB0214955
DdCP224Cterminus	PucC protein, putative	26076	DDB0217356
DdCP224Cterminus	Rubredoxin reductase	65595	DDB0216426
DdCP224Cterminus	Small aggregate formation protein	33344	DDB0191525
DdCP224Cterminus	Talin homologue	263623	DDB0219577
DdCP224Cterminus	WimA (Fragment)	221690	DDB0191321
DdCP224Nterminus	4-hydroxyphenylpyruvate dioxygenase	41607	DDB0231604
DdCP224Nterminus	6-phosphogluconate dehydrogenase	54404	DDB0215011
DdCP224Nterminus	Acetylornithine deacetylase	49469	DDB0191165
DdCP224Nterminus	Actin	45126	DDB0220456
DdCP224Nterminus	Actin15	41735	DDB0220460
DdCP224Nterminus	ADP/ATP translocase	31224	DDB0201558
DdCP224Nterminus	Ahcy13 protein (Adenosylhomocysteinase)	47908	DDB0191108
DdCP224Nterminus	Aldehyde reductase	33877	DDB0215363
DdCP224Nterminus	Alkyldihydroxyacetonephosphate synthase	70607	DDB0191146
DdCP224Nterminus	Beta-tubulin	51849	DDB0191169
DdCP224Nterminus	Bifunctional purine biosynthesis protein purH	59487	DDB0230095
DdCP224Nterminus	Calcium-dependent cell adhesion molecule-1	24154	DDB0191175
DdCP224Nterminus	Calcium-regulated actin bundling protein	33638	DDB0214810

Bait	Protein	Mass in Da	DictyBase ID
DdCP224Nterminus	Calmodulin	17151	DDB0214955
DdCP224Nterminus	CGI-81 protein	42209	DDB0204116
DdCP224Nterminus	Clathrin-adaptor medium chain apm 1	48851	DDB019110
DdCP224Nterminus	Cystathionine gamma-lyase	42896	DDB0191318
DdCP224Nterminus	Cytosolic aldolase	39189	DDB0231387
DdCP224Nterminus	DD-1 protein	22291	DDB0216234
DdCP224Nterminus	Dihydroxyacetone phosphate acyltransferase	146348	DDB0188248
DdCP224Nterminus	Discoidin I, D chain	25933	DDB0220114
DdCP224Nterminus	Discoidin II	28575	DDB0215382
DdCP224Nterminus	Elongation factor 1 beta	24364	DDB0191174
DdCP224Nterminus	Elongation factor 1-alpha (EF-1-alpha)	49662	DDB0191135
DdCP224Nterminus	Elongation factor 1-gamma (EF-1-gamma)	46927	DDB0185297
DdCP224Nterminus	Enolase	209969	DDB0218441
DdCP224Nterminus	Gamma-glutamyltranspeptidase	33875	DDB0203420
DdCP224Nterminus	GBP protein	57267	DDB0168276
DdCP224Nterminus	Glucose-6-phosphate dehydrogenase	56697	DDB0217233
DdCP224Nterminus	Glyceraldehyde 3-phosphate dehydrogenase	36780	DDB0185087
DdCP224Nterminus	GTP-binding nuclear protein RAN/TC4	24218	DDB0215409
DdCP224Nterminus	Hypothetical 40.0 kDa protein	90208	DDB0218270
DdCP224Nterminus	IfdA	44196	DDB0191262
DdCP224Nterminus	Inosine-5'-monophosphate dehydrogena	56767	DDB0230098
DdCP224Nterminus	Isocitrate dehydrogenase [NADP] cytoplasmic	46826	DDB0231401
DdCP224Nterminus	Microtubule-associated protein CP224	223812	DDB0189914
DdCP224Nterminus	Microtubule-associated protein EB1	51789	DDB0214955
DdCP224Nterminus	Myosin	239699	DDB0191444
DdCP224Nterminus	NAD-specific glutamate dehydrogenase	39168	DDB0206512
DdCP224Nterminus	Nucleolar protein	61199	DDB0186654
DdCP224Nterminus	Outer mitochondrial membrane protein porin	30158	DDB0185213
DdCP224Nterminus	PucC protein, putative	26076	DDB0217356
DdCP224Nterminus	Putative peroxisomal-coenzyme A synthetase	59158	DDB0205849
DdCP224Nterminus	Putative GTP-binding protein	74492	DDB0186513
DdCP224Nterminus	Putative methyltransferase	33440	DDB0189501
DdCP224Nterminus	Putative phosphoenolpyruvate carboxykinase	63084	DDB0231108
DdCP224Nterminus	Pyruvate kinase	48923	DDB0231421
DdCP224Nterminus	Rubredoxin reductase	65636	DDB0216426
DdCP224Nterminus	S-adenosylmethionine synthetase	42089	DDB0230070
DdCP224Nterminus	Serine hydroxymethyltransferase	49976	DDB0230072
DdCP224Nterminus	Small aggregate formation protein	33344	DDB0191525
DdCP224Nterminus	Transketolase glycolaldehydetransferase	61594	DDB0231244
DdCP224Nterminus	Ub52	15326	DDB0214925

Bait	Protein	Mass in Da	DictyBase ID
EB1	Actin 15	41735	DDB0220460
EB1	Actin act10	41747	DDB0220457
EB1	Myosin II heavy chain	243786	DDB0191444
EB1	Calmodulin	17151	DDB0214955
EB1	Discoidin II	28575	DDB0215382
EB1	Elongation factor 1-alpha (EF-1-alpha)	49662	DDB0191135
EB1	F-actin capping protein alpha subunit	31162	DDB0191243
EB1	Gamma-glutamyltranspeptidase	33875	DDB0203420
EB1	Glucose-6-phosphate dehydrogenase	56697	DDB0231285
EB1	Guanine nucleotide-binding protein beta subunit-like protein	36231	DDB0185122
EB1	Guanylyl transferase	32462	DDB0217857
EB1	Heat shock protein Hsp70Bb	70700	DDB0191168
EB1	Hexaprenyldihydroxybenzoate methyltransferase, mitochondrial precursor	36096	DDB0215313
EB1	Imidazoleglycerol-phosphate synthase subunit H-like	24362	DDB0215963
EB1	Microtubule-associated protein CP224	223812	DDB0189914
EB1	Microtubule-associated protein EB1	51789	DDB0214955
EB1	Myosin regulatory light chain	18323	DDB0185146
EB1	Arp2/3 complex 34 kDa subunit	32932	DDB0214935
EB1	PucC protein, putative	26076	DDB0217356
EB1	Rubredoxin reductase	65636	DDB0216426
EB1	Small aggregate formation protein	33344	DDB0191525
EB1	Vacuolar atp synthase subunit	26572	DDB0185070

2 Sequence of DdTACC

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1 CTTTTAATATTATTTCAATTAAAATTTGTTAAAACAATTAGTAATAAAATTGAAAATCAAATAGTAACTGAAATTAAAAAAAAACAATTTTTCTTTTTAA 100

101 AACATTATAAATCTAGTTTACAGTTTATAAAAAATATTTCAAAAAGTAATAAAATTATAGCGGATTTAGAAATTACAAAATCAAACCTTTTTGTTGGAAA 200

201 ATTAAGGAAAAGTCAATTATTGGGTTTATTTGGTTTAAATTCAATTTTAGGAACCCAATAAAAAAAATTTTAAATATATATTCCTTCTAGTTGTTCCTCAT 300

301 AATTACAAAA ATG AAA GGT TAA TAATTAAATAAAAATTTTGACCAAAAAAATCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTTTTTT 395

396 TTTTTGTTTTTTTTTTTTGTTTAAAAAATATTTATTATTAATTATTATCAAAATTGGTGTTATTTTGAGTTATAAAAAAAAAAAAAAAAA ATG AAT   491

492 GGT TAA TAATTATAAAAACAATTGTGAAGATTATTATTTTTTGTTTTTTTTTTATTTCTTATTTTTTTTATTTAATTTTAAATTAAATTAATTTTTTA 589

590 AAGATTTTAATAAACAAA ATG TAT AAA TAG TAAAAAAGATTTAAAAAAAAAAAAAATTTTAACCACCATAAAATACTTTGGTAAGTGGTG ATG  682

683 ATC AAT CCA TTA CAT ATG TGC TTT TTT TTT TTA ATT TTA TTT TTT TTA AAT TTT TTT TTT TAA ATTTTTTTTCTTTTTT 761

762 TTTTTTTTTGGTCACAATTATCATAATTAAAAAAAACAAAAAAAAAAAAAAAAAAAAAGCGAATTAGTTTTATTTTTA ATG TTT AAT AGA TAA AA 855

856 CAAAAACTAAAACTAAAAAACAAAAACTAAAACAAAAACAAAAACAAAAACAAGAAAAAACAATTTGTATTTTTATATATATTATTTATTTTTTTTTTTT 955

956 TTAAAAAATTTTATTTTTATAATAAATTTTTTTAAAATTTAATTA ATG GAT AAT GAA AAA TTA AAA AGA TTT CAA GAA AGA TAT    1039
1                                               M   D   N   E   K   L   K   R   F   Q   E   R   Y      13

1040 GAA CTT GAA TTA ATA GGT GTT GAA GAT AAT AAT GAT GAA GAT ATT GAA TAT TAT AGA TTT AAA GAA TCT TTA GAA  1114
14 E   L   E   L   I   G   V   E   D   N   N   D   E   D   I   E   Y   Y   R   F   K   E   S   L   E    38

1115 AAT GGT GGT GAC GGT ATT TCA GCC ATT CAA AAT GGT GGT GAT GGT GAT GGT GAT GGT AAT CAA GAT AAA AGT GAT  1189
39 N   G   G   D   G   I   S   A   I   Q   N   G   G   D   G   D   G   D   G   N   Q   D   K   S   D    63

1190 GCA GAT TTA GAG TAT GAG AGA CAA GCT CAA ATT GCA TTA CAA CAA CAA CAA GAG AAA CAA AGA CTA ATG GAA TTA  1264
64 A   D   L   E   Y   E   R   Q   A   Q   I   A   L   Q   Q   Q   Q   E   K   Q   R   L   M   E   L    88

1265 GAG ATT CAA AGA GAA TAT GAA AGA TTG GCA GCA GAA GGT CAA GCT GTT GAA CAA GAT TTC GAT TAT GAA GCT GAA  1339
89 E   I   Q   R   E   Y   E   R   L   A   A   E   G   Q   A   V   E   Q   D   F   D   Y   E   A   E    113

1340 GAA AGA AAG AGA CAA GAA GAA TCT AGA AAA AGA TTC CTA GAG AGA CAA AAT AGA AAT AAA CCT TCA CCA AAA AAG  1414
114 E   R   K   R   Q   E   E   S   R   K   R   F   L   E   R   Q   N   R   N   K   P   S   P   K   K    138

1415 AAA ACA CCT GAA GAA GAA GAA AAA GAA AGG ATA AAA TTA GAA AAA CAA AAA GAA AGA GAT CAA CAA AGA GAA TTA  1489
139 K   T   P   E   E   E   E   K   E   R   I   K   L   E   K   Q   K   E   R   D   Q   Q   R   E   L    163

1490 ATG AGA CAA GCA ATG AGT GGT AGT AGT AAT TCA AAG AAA GAA ACA CCA TTA GAA CAA CAA CAA CAA CAA CAA CAA  1564
164 M   R   Q   A   M   S   G   S   S   N   S   K   K   E   T   P   L   E   Q   Q   Q   Q   Q   Q   Q    188

1565 CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA  1639
189 Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q    213

1640 CAA CAA CAA CAA CAA ACA AAA CCA AAA TCA CCA TCA CCA CCT CCA CCA CAA CAA CAA GCA GAA GAA CCA ATT TCA  1714
214 Q   Q   Q   Q   Q   T   K   P   K   S   P   S   P   P   P   P   Q   Q   Q   A   E   E   P   I   S    238

1715 CAA CCT ACA TTA AAT GAT GAT GAA TAT GAA AGA CAA GCT CAA ATT GCA TTA CAA CAA CAA CAA GAG AAA CAA AGA  1789
239 Q   P   T   L   N   D   D   E   Y   E   R   Q   A   Q   I   A   L   Q   Q   Q   Q   E   K   Q   R    263

1790 CAG ATG GAA TTG GAG ATT CAA AGA GAA TAT GAA AGA TTA GCA GCA GAA GGT CAA GGT GTT GAA GAA AAT ATC GAT  1864
264 Q   M   E   L   E   I   Q   R   E   Y   E   R   L   A   A   E   G   Q   G   V   E   E   N   I   D    288

1865 TAT GAA GCT GAA CAA AGA AAA AGA GAA GAA GAA TCT AGA AAG AGA TTC TTG GAA AGA CAA AAT AAA TCA AAA GGT  1939
289 Y   E   A   E   Q   R   K   R   E   E   E   S   R   K   R   F   L   E   R   Q   N   K   S   K   G    313

1940 GCT GCA AAA CAA AAA ACA CCT GAA GAT GAA GAA AAG GAG AGA ATT AAA TTA GAA AAA CAA AAA GAA AGA GAT CAG  2014
314 A   A   K   Q   K   T   P   E   D   E   E   K   E   R   I   K   L   E   K   Q   K   E   R   D   Q    338

2015 CAA AGA GAA TTA ATG AAA CAG GCA ATG AGT GGT AGT GGT ACT CCA ACG AAA GAA TCA AAA CAA GAG GAA TCA AAA  2089
339 Q   R   E   L   M   K   Q   A   M   S   G   S   G   T   P   T   K   E   S   K   Q   E   E   S   K    363

2090 CCA TCA CAA AAC GAT CAA CCA ATT TCA AAA CCA AAA TCA CCA TCA CCA ATT CCG CCA CCA CAA CAA CAA CAA CAA  2164
364 P   S   Q   N   D   Q   P   I   S   K   P   K   S   P   S   P   I   P   P   P   Q   Q   Q   Q   Q    388

2165 CCA CCA CCA GAA GAA CCA ATT TCA CAA GCA CCA CCA ACA GAT GAT GAA TAT GAA AGA CAG GTT CAA ATT GCA TTA  2239
389 P   P   P   E   E   P   I   S   Q   A   P   P   T   D   D   E   Y   E   R   Q   V   Q   I   A   L    413

2240 CAA AAA CAA CAA GAA AAA CAA AAA CAG ATG GAA TTA GAG ATT CAA AGA GAA TAT GAA AGA TTA GCA GCA GAA GGT  2314
414 Q   K   Q   Q   E   K   Q   K   Q   M   E   L   E   I   Q   R   E   Y   E   R   L   A   A   E   G    438

2315 CAA GGT TTT GAA GAG ATC GAT TAT GAA GCA GAG GAA AAG AAA AGA CAA GAA GAA TCT AGA AAG AGA ATG TTG GAA  2389
439 Q   G   F   E   E   I   D   Y   E   A   E   E   K   K   R   Q   E   E   S   R   K   R   M   L   E    463

2390 AGA CAA AAT AAA TCA AAA GGT GCT GCA AAA CAA AAA ACA CCT GAA GAT GAA GAA AAA GAA AGA ATT AAA TTA GAA  2464
464 R   Q   N   K   S   K   G   A   A   K   Q   K   T   P   E   D   E   E   K   E   R   I   K   L   E    488

2465 AAA CAA AAA GAA AGA GAT CAA CAA AGA GAA TTA ATG AAA CAA GCA ATG GGT AGT GGT AAT AAT ACA CCA AAG AAG  2539
489 K   Q   K   E   R   D   Q   Q   R   E   L   M   K   Q   A   M   G   S   G   N   N   T   P   K   K    513

2540 GAA GAA CCA AAA CCA ATT GAA GAA CAA TCA AAA CCA CCA ACA TCC CAA CCT AAA TCT CAA TCA CCA CCA CCA CAA  2614
514 E   E   P   K   P   I   E   E   Q   S   K   P   P   T   S   Q   P   K   S   Q   S   P   P   P   Q    538

2615 AAA GTA GAA GAA CCA ATT TCA CAA GCA CCA CCA ACA GAT GAG GAA TAT GAA AGA CAA GCT CAA ATT GCA TTA CAA  2689
539 K   V   E   E   P   I   S   Q   A   P   P   T   D   E   E   Y   E   R   Q   A   Q   I   A   L   Q    563

2690 CAA CAA CAA GAG AAA CAA AGA CAG ATG GAA TTA GAG ATT CAA AGA GAA TAT GAA AGA TTA GCA GCA GAA GGT CAA  2764
564 Q   Q   Q   E   K   Q   R   Q   M   E   L   E   I   Q   R   E   Y   E   R   L   A   A   E   G   Q    588

2765 GGT GTT GAA CAA GAT TTC GAT TAT GAA GCT GAA CAA AGA AAA AGA GAA GAA GAA TCT AGA AAG AGA TTC TTG GAA  2839
589 G   V   E   Q   D   F   D   Y   E   A   E   Q   R   K   R   E   E   E   S   R   K   R   F   L   E    613

2840 AGA CAA AAT AAA TCA AAA GGT GCT GCA AAA CAA AAA ACA CCT GAA GAT GAA GAA AAG GAG AGA ATT AAA TTA GAA  2914
614 R   Q   N   K   S   K   G   A   A   K   Q   K   T   P   E   D   E   E   K   E   R   I   K   L   E    638

2915 AAA CAA AAA GAA AGA GAT CAA CAA AGA GAA TTA ATG AAA CAG GCA ATG AGT GGC GGT GGT GGT AAT GGT ACA CCA  2989
639 K   Q   K   E   R   D   Q   Q   R   E   L   M   K   Q   A   M   S   G   G   G   G   N   G   T   P    663

2990 AAG AAG GAA GAA CCA GAA GAA GTA TCA AAA CCA AAA GAA GTA TCA AAA CCA AAA GAA GTA TCA AAA CCA TCA TCA  3064
664 K   K   E   E   P   E   E   V   S   K   P   K   E   V   S   K   P   K   E   V   S   K   P   S   S    688

3065 TCA CCA TCA CCA CCA CCA CAA GTA GAA GAA CCA ATT TCA CAA GCA CCA CCA ACA GAT GAA GAG TAT GAA AGA CAA  3139
689 S   P   S   P   P   P   Q   V   E   E   P   I   S   Q   A   P   P   T   D   E   E   Y   E   R   Q    713

3140 GCA CAA CAA GCA TTG CAA CAA CAA CAA GAG AAA CAA AGA CAG ATG GAA TTA GAG ATT CAA AGA GAG TAT GAA AGA  3214
714 A   Q   Q   A   L   Q   Q   Q   Q   E   K   Q   R   Q   M   E   L   E   I   Q   R   E   Y   E   R    738
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3215 TTA GCA GCA GAA GGT CAA GGT GTT GAA GAA AAT ATC GAT TAT GAA GCT GAA GAA AGA AAG AGA CAA GAA GAA TCA  3289
739 L   A   A   E   G   Q   G   V   E   E   N   I   D   Y   E   A   E   E   R   K   R   Q   E   E   S    763

3290 AGA AAG AGA TTC CTA GAG AGA CAA AAT AAA TCA AAA AAC CCT GCA AAA CAA AGA ACA CCT GAA GAT GAA GAA AAA  3364
764 R   K   R   F   L   E   R   Q   N   K   S   K   N   P   A   K   Q   R   T   P   E   D   E   E   K    788

3365 GAA AGA ATT AAA TTA GAA AAA CAA AAA GAA AGA GAT CAA CAA AGA GAA TTA ATG AAA CAA GCA ATG AGT GGT GGC  3439
789 E   R   I   K   L   E   K   Q   K   E   R   D   Q   Q   R   E   L   M   K   Q   A   M   S   G   G    813

3440 GGT GGT ACT CCA AAG AAA GAG ACA CCA AAA TCA CAA GAA GAA CAA TCA AAA CCA ATT GAA GAA CAA TCA AAA CCA  3514
814 G   G   T   P   K   K   E   T   P   K   S   Q   E   E   Q   S   K   P   I   E   E   Q   S   K   P    838

3515 AAA TCT CCA CCA CCA CAA CAA CAA CAA CAA CAA ATA GAG GAG ACA ACA TCA CAA CCA ACA TCA AAT GAT GAT GAA  3589
839 K   S   P   P   P   Q   Q   Q   Q   Q   Q   I   E   E   T   T   S   Q   P   T   S   N   D   D   E    863

3590 TAT GAA AGA CAG GTT CAA ATT GCA TTA CAA AAA CAA CAA GAA AAA CAA AGA CAG ATG GAA TTA GAG ATT CAA AGA  3664
864 Y   E   R   Q   V   Q   I   A   L   Q   K   Q   Q   E   K   Q   R   Q   M   E   L   E   I   Q   R    888

3665 GAA TAC GAA AGA TTA GCA GCA GAA GGT CAA GGT GTT GAA GAA AAT ATC GAT TAT GAA GCT GAA GAA AAG AAA AGC  3739
889 E   Y   E   R   L   A   A   E   G   Q   G   V   E   E   N   I   D   Y   E   A   E   E   K   K   S    913

3740 CAA GAA GAA TCT AGA AAG AGA ATG TTG GAA AGA CAA AAT AAA GCA AAA GGT GCA GCA AAA CAA AAA ACA CCT GAA  3814
914 Q   E   E   S   R   K   R   M   L   E   R   Q   N   K   A   K   G   A   A   K   Q   K   T   P   E    938

3815 GAT GAA GAA AAA GAA AGA ATT AAA TTA GAA AAA CAA AAA GAA AGA GAT CAA CAA AGA GAA TTA ATG AGA CAA GCA  3889
939 D   E   E   K   E   R   I   K   L   E   K   Q   K   E   R   D   Q   Q   R   E   L   M   R   Q   A    963

3890 ATG AGT GGT GTA AGT AAT GGT GAA TCA CCT GTA AAG AAA GAT AAA CAA TCA TCA TCA TCA TCA TCA CAA AAA CCA  3964
964 M   S   G   V   S   N   G   E   S   P   V   K   K   D   K   Q   S   S   S   S   S   S   Q   K   P    988

3965 TCA GAA GAA GAA AGT AAA TCT GAT CAA CCA ACA ACA AAA TCT AAA TCC CCA TCA CCT CAA CCA ACA GAA AAT AAT  4039
989 S   E   E   E   S   K   S   D   Q   P   T   T   K   S   K   S   P   S   P   Q   P   T   E   N   N   1013

4040 AAT AAT AAT AAT AAT AAT AAT AGT AAT GAA GAT GAT GAA TAT GAA AGA AAA GCA GCA ATT GCA TTA AAA CAA CAA  4114
1014 N   N   N   N   N   N   N   S   N   E   D   D   E   Y   E   R   K   A   A   I   A   L   K   Q   Q   1038

4115 CAA GAA AAA CAA AGA CAA ATG GAA TTA GAA CTT CAA AGA GAA TAT GAA AGA TTG GCA GCA GAG GAG GGT AAA TCA  4189
1039 Q   E   K   Q   R   Q   M   E   L   E   L   Q   R   E   Y   E   R   L   A   A   E   E   G   K   S   1063

4190 CCA GAA CCA ACA CCT GAA GAA ATT GAG GCA GAA CAA AAG AAA AAA GAA GAA GAA ATG AAA AGA AGA TTC CAA GAA  4264
1064 P   E   P   T   P   E   E   I   E   A   E   Q   K   K   K   E   E   E   M   K   R   R   F   Q   E   1088

4265 AGA CAA AAA CAA TCA ACA CAA AAA CAA AAA ACA CCT GAA GAT GAA GAA AAG GAA AGA ATT AAA TTA GAG AAA CAA  4339
1089 R   Q   K   Q   S   T   Q   K   Q   K   T   P   E   D   E   E   K   E   R   I   K   L   E   K   Q   1113

4340 AAA GAA AGA GAT CAA CAA AGA GAA TTA ATG AAA CAA ACA ATG AGT GGT GGT GAC CCA AAT GGT AAA TCA CAA CAA  4414
1114 K   E   R   D   Q   Q   R   E   L   M   K   Q   T   M   S   G   G   D   P   N   G   K   S   Q   Q   1138

4415 CAA AAA CAA TCT CAA CCA TTA CCA GAA GAA CCA CCC AAA CCA ATT TCA CAA CCT CAA ACA ACT CAA CCA AAA CCC  4489
1139 Q   K   Q   S   Q   P   L   P   E   E   P   P   K   P   I   S   Q   P   Q   T   T   Q   P   K   P   1163

4490 CTA TCT CCA TCA CAA CAA CAA CAA CAA CAA CAA CAA CAA CCA CAA CCA CAA CAA CAA CCT CAA TCA CAA CCA  4564
1164 L   S   P   S   Q   Q   Q   Q   Q   Q   Q   Q   Q   Q   P   Q   P   Q   Q   Q   P   Q   S   Q   P   1188

4565 CAA CTT CAA TCA CAA CCA CAA TCA CAA CAA CAT CAA CCA ATT TCA TCA CAA GAT GGA TTT AAT TTA CAA TCT CAA  4639
1189 Q   L   Q   S   Q   P   Q   S   Q   Q   H   Q   P   I   S   S   Q   D   G   F   N   L   Q   S   Q   1213

4640 CAA TAT TAT CAA CAA ATG CAA CAA CAA CAA CAA CAA TTT TAT CAA CAA TTT AAT CAA TAT AAT CAT CCA TCA AGT  4714
1214 Q   Y   Y   Q   Q   M   Q   Q   Q   Q   Q   Q   F   Y   Q   Q   F   N   Q   Y   N   H   P   S   S   1238

4715 CCA AAT CAT CAT AAC CAA CAA CAA CAA CCA ATG TAT CAT CAA CAA AAT CAA TCA CCA TCA CAT AAT AAT AAT AAT  4789
1239 P   N   H   H   N   Q   Q   Q   Q   P   M   Y   H   Q   Q   N   Q   S   P   S   H   N   N   N   N   1263

4790 AGT AAT AAT AGT AGT AAT ATA TCA TCA CCA CCA CCA CCA ACA CAT CAA AAT ATG TAT CCA TAC CCA CAT CAA TTT  4864
1264 S   N   N   S   S   N   I   S   S   P   P   P   P   T   H   Q   N   M   Y   P   Y   P   H   Q   F   1288

4865 TAT TAT CCA CCA CCA CAT CAA AAC ATG TTT ACT CAA GAA GAT ATT GAT CGT TTA TTA GAA AAA CAA AAA AAA GAA  4939
1289 Y   Y   P   P   P   H   Q   N   M   F   T   Q   E   D   I   D   R   L   L   E   K   Q   K   K   E   1313

4940 ATT TTA AAA CAA GTA AGT TAT TAT TTT ATT ATT TAT TTT ATT TTA TTT TAT TTT TTT TTT TTT ATT TTA TTT ATT  5014
1314 I   L   K   Q                                                                                       1317

5015 TTT ATT AAT TTA ATA AAA AAA AAA AAA AGT GTT CAG ATG AAA ATG CAA TTT TAT CAT TAG AATTAGAAGATTTACAATTA 5094
1318                                     C   S   D   E   N   A   I   L   S   L   E   L   E   D   L   Q   L  1334

5095 AAATTAGAAAAAGAACAAAGAAATTGTTTATCATTAAAAGGTATATTATCACAAT ATG AAG CAA CAA TGA AGAAA ATG ATT GAT CAA    5181
1335 K   L   E   K   E   Q   R   N   C   L   S   L   K   G   I   L   S   Q   Y   E   A   T   M   K   K   M   I   D   Q      1363

5182 CCA GCA CCA GTA CCA CAA AAC TCT GCA AAA CTT AGT GAA GCA TTA TTA GAT AAT GAA CAA TTG AGA AAA CAA ATC  5256
1364 P   A   P   V   P   Q   N   S   A   K   L   S   E   A   L   L   D   N   E   Q   L   R   K   Q   I   1388

5257 ATT GAT TTA AAG GAA TCC AAT TTA CAA GCC AAC AAA GAG AAT CAA ATT CAT GTC TCT AAA TTT AAT GCA CTA AAA  5331
1389 I   D   L   K   E   S   N   L   Q   A   N   K   E   N   Q   I   H   V   S   K   F   N   A   L   K   1413

5332 GCA CAT GCC GAA GCA AAA TTA GAT AGT GCC TCT GAA CAA TTA ACA AAA TTG AAA GAA GTT GCC AAA AAA GAA ATT  5406
1414 A   H   A   E   A   K   L   D   S   A   S   E   Q   L   T   K   L   K   E   V   A   K   K   E   I   1438

5407 GAA GTA CTA ACT ATT AAA TTA AAT CGT TCA GAG TCA AAA GTT TCA ATT AAA GAA AAA GAA AAT GAT GAA TTG GTT  5481
1439 E   V   L   T   I   K   L   N   R   S   E   S   K   V   S   I   K   E   K   E   N   D   E   L   V   1463

5482 AAA TTA TGT GAT GAA TTA ATT TTT AAA TTA CAA ACT AAT CAA AAA TAA AACAAAAAATAAAATAAATAAAAAAAATAACTACAA 5565
1464 K   L   C   D   E   L   I   F   K   L   Q   T   N   Q   K   *                                       120

5566 TATTTTTTAAAAAAAAAAAAATAAAATAATA ATG ATT TTA AAA CAA ATA ATT ATC ACT GTA ATA AAA TAA ATAAAAAAGTTTAATA 5651

5652 TTTAAATTATAAAAAAAAAAAAAAAAAATTATTATTATTATTATTATTGTTATTATTATTATTAAACAAACTATCATTTATAGAAATAAAAAAAAAAAAAC 5751

5752 AATAATATTCTCATTGAGTTTATTATTATTTTTTATTTATTTATTTTTTTTATTTTTTTATTTTTTTATTTATTTATTTTTTTTATTTTAATTATT 5851

5852 TTAAAAAAAAGTTTTTTTAAAATTTATTTTAAAATTTATTTTTTTAATTTAGTTATAAGATAGAAACCAAGAGCAATTGCACCAACAATTGAAGTTGCAA 5951

5952 TTGCCACTTTAGCAACAGGTCCAT ATG GTA CGA TAG ATTTATTTTGATTTGAAGTGTTATCATCATCATCGTCAGAGTCGTCAGAGTCTGAATCA 6046

6047 GATTCTGAATCAGATTCACTTTCTTCTTCACTATCTGATTCACTATCTGATTCACTATCAGAGGAAGTATTTTCACCTTTTTGATCTTCTTTACCAACAT 6146

6147 TTAAAATACTATCTAAAGCATCTCTCATTGGTTG ATG ATC TGG TTC AAT TAA AAGACCTTTTTCATAATATTTG ATG GCT TCA TCT    6232

6233 TTT TGA TTTAATTTTTCTAAAGCTTGACCAGCTCT ATG ATA ACC TTT AAC CCA ATT AGC TTC CAA TTT AAT TGA TTCCA ATG  6314

6315 CAT CTT GAA GTG ATT CAG CAA ATA ATC CAA ATT TAT AAT ATG AAA TTG ATC TAT TAC TAT ACA AGA GAT GTG ATG  6389

6390 GTT TAA TTGAA ATG GCT TTT GAA TAA CTTTCGATTGCATCAAAGTAAATATT ATG TTT AAA GTA TTC ATT ACC TTC AAG TTT 6471

6472 ATA AAA GTT TGC TAA TTCTGCTTGTGATAATATATTTTCATCCTCTTTTTCAACTTCT                                      6529
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Sequence of DdTACC Sequence of the open reading frame is depitced along with 3' and 5' utrs. Respective amino acid sequences are represented by single-letter code. Location of primer

pairs used for amplification of the GFPTACC domain construct is marked by underlining the sequence. Sequence was taken from www.dictybase.org.

Curriculum vitae

Katrin Veronika Koch

Geburtsdatum	11.06.1976 in München
Familienstand	Ledig

Schul- und Hochschulbildung

04/2003-10/2006	**Promotion** am A. Butenandt Institut für Zellbiologie, LMU München Leitung: Prof. Dr. R.Gräf, Abt. Prof. Dr. M.Schliwa Betreuung: Prof. Dr. M. Bölker, Philipps-Universität Marburg
04/2001 - 09/2002	**Diplomarbeit** in der Arbeitsgruppe von PD. Dr. R. Fischer, Abt. Biochemie, Prof. Dr. R. Thauer am Max-Planck-Insitut für terrestrische Mikrobiologie, Marburg
10/1998 - 02/2001	**Hauptstudium** an der Philipps-Universität Marburg
08/1997 - 07/1998	**Auslandsstudium** an der University of Alberta, Edmonton, Kanada
10/1995 - 07/1997	**Grundstudium** der Biologie an der Philipps-Universität Marburg (vorgezogenes Vordiplom)
08/1986 - 06/1995	**Gymnasium** am Moltkeplatz, Krefeld
08/1982 - 06/1986	**Grundschule** an der Eichendorffstraße, Krefeld

Stipendien

seit 09/2005	Mitglied des selbstorganisierten Doktorandenkollegs „Protein Dynamics in Health and Disease“ im **Elitenetzwerk Bayern**;
09/10/2004	**Tagungsteilnahme** am 6th Young Scientists Meeting „Cytoskeletal Dynamics“, Heidelberg (unterstützt von Zeiss)

Dank

In der besonderen Situation dieser Arbeit haben viele Menschen zum Gelingen mit beigetragen:
Zuallererst natürlich Ralph Gräf; ich hatte alle Freiheit, die ich wollte und wusste in ihm doch stets einen Ansprechpartner mit unzähligen Tipps und Ratschlägen. Auch als er das Labor verlassen hatte, blieb er stets ansprechbar und hilfsbereit. Ich freue mich, dass nun in Potsdam noch mehr Doktoranden in diesen Genuss kommen können!

Manfred Schliwa danke ich für meinen Arbeitsplatz am A. Butenandt Institut. Die Diskussionen mit ihm waren sehr anregend und es tat gut zu wissen, dass seine Tür immer offen steht. Mit der Auswahl für das EliteNetzwerk hat er mir eine große Freude gemacht!

Besonders danke ich auch Michael Bölker für die Übernahme meiner Betreuung in Marburg. Die Gespräche mit ihm und seiner Arbeitsgruppe waren mir nie Pflicht, sondern stets Vergnügen, ein Austausch fast wie auf einer kleinen Tagung.

In der „meisterlosen“ Zeit habe ich mich besonders über die „Adoption“ von den Schleichers gefreut. Durch die Dictyseminare und die vielen netten Menschen, die ich immer fragen konnte, fühlte ich mich nie „allein auf (dem) weiter(en) Flur“.
Mit Thi Hieu Ho hatte ich das große Glück, die beste aller TAs an meiner Seite zu haben! Sie war mir eine große Hilfe und durch ihre Freundlichkeit war unser Labor immer ein warmer Ort. Auch allen anderen Mitgliedern unseres Labors möchte ich danken. Wie wohl man sich fühlt hängt vor allem von den Menschen ab: Julia Kleylein-Sohn, Markus Rehberg, Andrea Hestermann, Christine Zoglmeier, Matthias Samereier, Sebastian Weidenthaler und Irene Schulz (vielen Dank für die Hilfestellung bei Methoden, vor allem natürlich der Konstruktion von Trockeneis-vulkanen!).
In meinem Kampf mit dem Y2H-screen hatte ich auch einige Helfer: von Lucia Driller bekam ich eine sehr gründliche Einführung, die durch Hans Faix’ herzhaftes „passt scho!“ perfekt ergänzt wurde. Renate Dombi möchte ich für ihre Hilfsbereitschaft und die tatkräftige Unterstützung bei dem Versuch, den über 800 Klonen Herr zu werden, ganz herzlich danken!
Auch die anderen Institutsmitglieder möchte ich nicht vergessen: was wären wir ohne Maria Beer und Frau Lorenz! Bettina Ebbing danke ich für die schöne Schreibtischnachbarschaft. Und die Besuche von Usch Euteneuer brachten immer gute Gespräche mit sich.

Sehr dankbar bin ich für die Unterstützung durch meine Familie:
Mein Bruder Kristof half mir beim sprachlichen Feinschliff, mein Vater war als „alter“ Naturwissenschaftler in vielen Fällen ein guter Ratgeber und sicher auch Vorbild und meine Mutter schuf mir in den letzten Wochen durch ihre Pflege eine perfekte Arbeitsumgebung.
Die Hochs und Tiefs der Arbeit bekam sicher mein Freund Florian Pfütze am deutlichsten mit, ihm danke ich sehr für seine Geduld und liebevolle Aufmunterung und schon mal im Voraus für’s Händchenhalten beim Drucken!